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(54) Title: CLONING AND EXPRESSION OF A ACETYLCHOLINE-GATED ION CHANNEL RECEPTOR SUBUNIT

(57) Abstract

The present invention provides isolated nucleic acids encoding alpha9 nicotinic acetylcholine receptor subunit and receptor subunit encoded thereby. Also provided are vectors containing the invention nucleic acids, host cells transformed therewith, alpha9 nicotinic acetylcholine receptor subunit and functional nicotinic acetylcholine receptors comprising at least one alpha9 subunit expressed recombinantly in such host cells as well as transgenic non-human mammals that express the invention receptor subunit and mutants thereof. Receptors of the invention comprise at least one alpha9 nicotinic acetylcholine subunit and form cationic channels activated by acetylcholine, but blocked by nicotine and muscarine. The invention also provides methods for identifying compounds that modulate the ion channel activity of the functional invention receptors containing at least one invention subunit.

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CLONING AND EXPRESSION OF A
ACETYLCHOLINE-GATED ION CHANNEL RECEPTOR SUBUNIT

BACKGROUND OF THE INVENTION

Intercellular communication is essential for the function of multicellular systems. Ion channel proteins, as mediators of information transfer in the brain, 5 endocrine system, enteric nervous system and neuromuscular junction, modulate ion fluxes that produce voltage changes across cell membranes and simultaneously act as sensors of physiological signals, for example, changes in ligand concentrations and in transmembrane voltage. Ligand-gated 10 ion channels provide for rapid dialogue between cells of the central nervous system, converting a chemical neurotransmitter signal released from one cell into an electrical signal that propagates along the cell membrane of a target cell. Ligand-gated ion channels are multimeric 15 protein complexes with component subunits encoded by related genes.

At the present time, numerous families of ligand-gated receptors have been identified and

characterized on the basis of sequence identity. Those which form cationic channels include, for example, excitatory nicotinic acetylcholine receptors (nAChRs), excitatory glutamate-activated receptors, the 5-HT₃ 5 serotonin receptor, the ATP receptor and the sarcoplasmic ryanodine receptor. Those which form anionic channels include, for example, the inhibitory GABA and glycine-activated receptors.

The neurotransmitter acetylcholine (ACh) 10 activates two pharmacologically different receptor types: nicotinic acetylcholine receptors (nAChR) from the ligand-gated ion channel superfamily and muscarinic acetylcholine receptors (mAChR) from the G-protein coupled receptor superfamily (Taylor, A. Goodman-Gilman, T.H. Rall, 15 A.S. Nies and P. Taylor, eds. (New York:Pergamon Press), pp. 166-186,1990); (Taylor, A. Goodman-Gilman, T.H. Rall, A.S. Nies and P. Taylor, eds. (New York:Pergamon Press), pp. 122-149,1990). A number of pathologies and/or disease conditions are associated with nAChRs, such as, for 20 example, myasthenia gravis, schizophrenia, Alzheimer's disease, Tourette's disease and nicotine addiction. Biochemical and electrophysiological data have shown that nicotinic and muscarinic receptors are functionally distinct entities. (Bonner, et al., *Science*, 237, 527-532, 25 1987). Whereas nAChRs are pentamers composed of related protein subunits that span the plasma membrane four times, mAChRs are formed by a single polypeptide chain which is postulated to span the plasma membrane seven times.

Nicotinic acetylcholine receptors, glycoproteins 30 composed of five subunits, transduce the binding of acetylcholine in the cationic channel. The five receptor subunits form a pseudosymmetric ring around a central

channel. Neuronal nicotinic AChRs (NnAChRs) mediate neurotransmission at many central and peripheral synapses, and comprise two subunit types (alpha and beta) encoded by 10 different neuronal genes. Expression of particular 5 combinations of subunit RNAs in oocytes yields biophysically distinct channels that are distinguished pharmacologically on the basis of ligands that modulate such channels.

Recombinant DNA technology has enabled the 10 identification of the vertebrate muscle nAChR subunits alpha1, beta1, gamma, delta and epsilon and the neuronal subunits alpha2, alpha3, alpha4, alpha5, alpha6, alpha7, alpha8, beta2, beta3 and beta4 (rat nomenclature). Various 15 combinations of these subunits produce functional recombinant receptor-channel complexes that are activated by both ACh and nicotine. The nAChR at the neuromuscular junction is thought to have a $(\alpha 1)_2\beta 1\gamma\delta$ stoichiometry (Galzi, et al., *Annu. Rev. Pharmacol.*, 31, 37-72, 1991). In contrast, the neuronal nAChR subunits alpha2, alpha3 and 20 alpha4 lead to the assembly of functional nAChRs in concert with either beta2 or beta4 (Boulter, et al. *Proc. Natl. Acad. Sci. USA*, 84, 7763-7767, 1987; Ballivet, et al., *Neuron*, 1, 847-852, 1988; Wada, et al., *Science*, 240, 330-334, 1988; Deneris, et al., *Neuron*, 1, 45-54, 1988; 25 Duvoisin, et al., *Neuron*, 3, 487-496, 1989; Couturier, et al., *J. Biol. Chem.*, 265, 17560-17567, 1990), while the neuronal alpha7 and alpha8 subunits can form functional nAChRs in the absence of any other subunit (Couturier, et al., *J. Biol. Chem.*, 265, 17560-17567, 1990; Seguela, et 30 al., *J. Neurosci.*, 13, 596-604, 1993; Gerzanich, et al., *Molec. Pharmacol.*, 45, 212-220, 1994).

Given the existence of ten distinct nicotinic acetylcholine subunit genes, numerous combinations of subunits producing functional receptors are possible. In 35 spite of the numerous combinations of subunits which can be

prepared from previously cloned genes, the properties of the native nAChRs do not always match those of recombinant receptors (Sargent, *Annu. Rev. Neurosci.*, 16, 403-443, 1993). For example, the cholinergic receptors present in 5 bovine chromaffin cells and in rat and chick cochlear hair cells exhibit a pharmacological profile that does not fit any combination of known subunits (Shirvan, et al., *Proc. Natl. Acad. Sci. USA.*, 88, 4860-4864, 1991; Housley, et al., *Proc. R. Soc. Lond. B*, 244, 161-167, 1991; Fuchs, et 10 al., *Proc. R. Soc. Lond. B*, 248, 35-40, 1992; Erostegui, et al., *Hearing Res.*, 74, 135-147, 1994), thus suggesting the existence of additional, as yet unidentified subunits.

Thus, a need exists for identifying additional members of the nicotinic acetylcholine receptor 15 superfamily, and characterizing such nAChR subunits, as well as functional receptors assembled therefrom, which includes elucidation of the nature of assembly of various subunits in the production of a functional receptor (i.e., a subunit assembly containing ligand binding sites and a 20 ligand-gated transmembrane channel), and the relationship between the structure of the subunit assembly and the pharmacological profile of the corresponding receptor. The present invention satisfies these needs and provides related advantages as well.

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SUMMARY OF THE INVENTION

The present invention provides isolated nucleic acids encoding alpha9 nicotinic acetylcholine receptor (nAChR) subunit, isolated receptor subunit protein encoded thereby as well as recombinately expressed alpha9 nicotinic 30 acetylcholine receptor (nAChR). Further provided are vectors and probes containing such nucleic acids, host cells transformed with such nucleic acids, antisense oligonucleotides and compositions containing such oligonucleotides, antibodies that specifically bind to

invention receptors and compositions containing such antibodies as well as transgenic non-human mammals.

The alpha9 nAChR subunits of the invention form a cationic receptor channel complex which is activated by 5 acetylcholine and is permeable to cations, including calcium. Functional alpha9 nACh receptors of the invention may be expressed as homomeric receptors, i.e., only one type of subunit is required for function, or invention receptors may be expressed as heteromeric receptors wherein 10 more than one type of subunit is required to form a functional receptor. Additionally, the invention provides methods for identifying compounds that modulate activity of the invention receptors, or the activity of nucleic acid encoding such receptors.

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BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the nucleotide and the amino acid sequence of the cDNA clone coding for the alpha9 nAChR subunit. The amino acid sequence is shown below the nucleotide sequence. Cleavage of the signal peptide is 20 predicted at amino acid position 1 (Von-Heijne, *Nucl. Acid. Res.*, 14, 4683-4691, 1986). Amino acids encoding the signal peptide are assigned negative numbers. Nucleotides are numbered in the 5' to 3' direction, starting with the first nucleotide of the codon for the putative N-terminal 25 residue of the mature protein. Nucleotides on the 5' side of amino acid residue 1 are indicated by negative numbers. Arrowheads indicate the intron location determined by genomic sequencing. Membrane spanning regions are underlined. The sequence information presented in Figure 30 1 is also presented in PatentIn format in SEQ ID NOS 1 and 2.

Figure 2A shows the restriction map for the alpha9 subunit gene, and Figure 2B shows a partial

restriction map for overlapping genomic clones, M6 and MNANO, spanning the entire coding sequence of the alpha9 subunit gene. *Nco*I and *Nhe*I restriction sites not mapped in pMNANO, and *Sac*I restriction sites are not mapped in 5 pM6.

Figure 3 shows the alignment of amino acid sequences for known nAChR alpha subunits. All sequences correspond to rat subunits, except for alpha8 which is a chick subunit. Identical residues in all sequences are 10 presented as white letters in a black background. Spaces are introduced to maximize homologies. Predicted signal peptides and the four potential membrane spanning regions are indicated. Asterisks denote cysteine residues 127, 141, 191 and 192 (alpha9 numbering for the mature peptide, 15 absent the 28 amino acid residues comprising the signal peptide) conserved in all nAChR alpha subunits.

Figures 4A and 4B show electrophysiological responses of alpha9 injected oocytes to cholinergic agonists. Figure 4A shows current responses elicited by 20 ACh, nicotine, muscarine, 1,1-dimethyl-4-phenylpiperazinium (DMPP), and oxotremorine-M (OXO-M) in oocytes injected with alpha9 cRNA and held at -50 mV under voltage-clamp.

Figure 4B shows concentration-response curves to ACh, DMPP and OXO-M. Values represented are the mean and 25 standard error of the mean of peak current values obtained in at least four oocytes per drug. Error bars are not shown when the standard error is smaller than the symbol. Responses from each cell were normalized to the maximal current evoked by ACh. The Hill equation ($EC_{50} = 9.7 \mu M$; 30 slope = 1.3) was fitted to the ACh concentration-responsive curve.

Figures 5A and 5B show the blockage of ACh responses in alpha9 injected oocytes by various

antagonists. Inhibition curves were performed by the coapplication of 10 μ M ACh and increasing concentrations of either (-) nicotine or (+) muscarine (see Figure 5A) and strychnine, d-tubocurarine (d-TC) or atropine (see Figure 5B). Responses are expressed as the percentage (%) of the control current evoked by 10 μ M ACh. The mean and standard error of the mean of values obtained in at least four different oocytes per drug are shown. Error bars are not shown when the standard error is smaller than the symbol.

10 Figure 6 shows the sensitivity of ACh-evoked currents in alpha9 injected oocytes to α - and κ -bungarotoxin. Representative current responses to 100 μ M ACh recorded at a holding potential of -50 mV are shown. Oocytes were preincubated with α -bungarotoxin (α -BTX, A) or
15 κ -bungarotoxin (κ -BTX, B) for 30 minutes before the application of the second test concentration of ACh.

20 Figures 7A through 7C show the voltage-dependence of the ACh-evoked currents in alpha9 injected oocytes and Ca^{2+} permeability of the recombinant alpha9 receptor. In Figure 7A the current-voltage relationship of ACh-evoked currents in alpha9-injected oocytes was determined by applying a voltage ramp (2 second duration, +50 mV to -120 mV) during the plateau phase of the current response. The traces are representative of those obtained in four
25 different oocytes.

Figure 7B shows representative current traces elicited by 100 μ M ACh in alpha9-expressing oocytes before and after the injection of 50nl of 20mM 1,2-bis (2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA).

30 Figure 7C shows ACh-evoked currents in alpha9-injected oocytes held at -10 mV under voltage-clamp and bathed with a Ringer's solution containing 350mM NaCl.

Figure 8 shows detection of alpha9 transcripts in the rat cochlea. Amplification reactions were performed as described in Example VI, using cDNA transcribed from total RNA as template and alpha9 specific primers. Resolution of 5 the amplified products in a 1.5% agarose gel stained with ethidium bromide is shown. An aliquot of 10 μ M of each reaction mixture was loaded per lane. Lane 1, DNA ladder; lane 2, no DNA template; lane 3, amplified product from 10 olfactory epithelium cDNA; lane 4, amplified product from olfactory epithelium cDNA; lane 5, amplified product from sciatic nerve cDNA.

Figures 9A through 9F show the results of *in situ* hybridization of sagittal sections of rat embryos and coronal sections of adult brains and identification and 15 transcript localization of alpha9. Figures 9A and 9B show the presence of alpha9 transcripts in the hypophyseal gland, the olfactory epithelium, the sternohyoid muscle and the tongue, of a rat embryo at stage E16. Figures 9C and 9D show a high magnification view of the pituitary in a rat 20 embryo at stage E16 where alpha9 transcript is located in the pars tuberalis but not the pars distalis or the pars nervosa. Figures 9E and 9F show the presence of alpha9 transcripts in the pars tuberalis of the adult rat brain.

DETAILED DESCRIPTION OF THE INVENTION

25 Molecular cloning studies have demonstrated structural and functional diversity in nicotinic acetylcholine receptors (nAChRs). To date, seven alpha subunits (alpha2 to alpha8) and three beta subunits (beta2 to beta4) have been characterized in the nervous system of 30 vertebrates. The present invention describes the identification and functional characterization of a new member of this family of receptor subunit genes that are activated by the neurotransmitter acetylcholine (ACh). The new member is designated alpha9. The molecular structure

of alpha9 indicates that it belongs to the ionotropic (nicotinic) rather than to the metabotropic (muscarinic) ACh receptor family. However, the mixed nicotinic-muscarinic properties of the recombinant alpha9 receptor 5 differ from the pharmacological profile of all known functional nicotinic receptors.

Isolation and identification of the novel nAChR subunit gene of the present invention was accomplished by screening a rat genomic library using a rat nAChR alpha7 10 subunit cDNA as probe. DNA sequence analysis revealed that one isolated genomic clone encoded a protein with significant amino acid sequence identity with members of the ligand-gated ion channel gene superfamily. Its homology to known subunits revealed that it was more 15 related to nAChR subunits than to GABA_A, glycine or 5-HT₃ receptor subunits. The presence of conserved contiguous cysteine residues in the extracellular domain, which are a hallmark of all nAChR alpha subunits and are thought to be part of the acetylcholine binding domain (Popot and 20 Changeux, *Physiol. Rev.* **64**, 1162-1193, 1984) suggested that this gene encoded a nAChR alpha subunit. Therefore, in accordance with current nomenclature, this newly discovered subunit has been designated the alpha9 subunit of the nAChR gene family.

25 A polymerase chain reaction (PCR) fragment derived from the isolated genomic clone was used to screen a rat olfactory epithelium cDNA library. Four independent cDNA clones were isolated, one of which contained a 1937 bp insert encoding an open reading frame for the alpha9 30 subunit. The nucleotide and deduced amino acid sequences are shown in Figure 1 (and are also presented in Patentin format in SEQ ID NOS 1 and 2). The full length alpha9 cDNA encodes a mature protein of 451 amino acid residues, preceded by a leader sequence of 28 residues. It contains 35 all the features characteristic of other members of the

nAChR gene family, including four hydrophobic regions which predict potential membrane spanning regions, MSR I to IV (Kyte and Doolittle, *J. Mol. Biol.*, 157, 105-132, 1982), and cysteine residues at positions 127, 141, 191 and 192 5 (alpha9 numbering for the mature peptide, absent the 28 amino acid residues comprising the signal peptide) which are present in all nAChR alpha subunits.

The full-length alpha9 cDNA was used as a probe to screen two mouse genomic libraries constructed in phage 10 vectors lambdaDASH II and lambdaFIX II. Two overlapping genomic clones were obtained (see Figure 2). These clones, spanning the entire coding sequence of the alpha9 subunit gene, were cloned into plasmid vectors and the alpha9 subunit gene structure was determined by sequencing across 15 the intron-exon boundaries. The intron-exon boundaries of the alpha9 gene are indicated in Figure 1. The gene consists of five exons and has an intron-exon structure that differs from that of all known nAChR genes (Noda, et al., *Nature*, 305, 818-823, 1983; Nef, et al., *EMBO J.*, 7, 20 595-601, 1988; Wada, et al., *Science*, 240, 330-334, 1988; Buonanno, et al., *J. Biol. Chem.*, 264, 7611-7616, 1989; Boulter, et al., *J. Biol. Chem.*, 265, 4472-4482, 1990). For example, in contrast to other nAChR subunit genes where the 25 intron-exon boundaries of the first four exons are conserved, exons III and IV of the alpha9 gene are fused.

The alpha9 cDNA clone was sequenced and the sequence compared with sequences of other nAChR alpha subunits (see Figure 3). Based on sequence similarity, the alpha9 subunit appears to be a distant member of the nAChR 30 subunit gene family. It is as distinct from the neuronal alpha7-alpha8 subfamily (38% amino acid sequence identity) as it is from the neuronal alpha2-alpha6 (36-39%) subfamily or from the muscle alpha1 subunit (37%). Although alpha9 shares the most highly conserved sequence elements with 35 other members of the family, some amino acid residues

differs from those found invariant in the other alpha subunits. For example, the conserved hydrophobic residues Phe-99 and Val-230 (alpha9 numbering for the mature peptide, absent the 28 amino acid residues comprising the 5 signal peptide) are changed to the polar residues Ser-99 and Ser-230 in the alpha9 protein and the conserved positively charged residue Lys-144 is substituted by the non-charged residue Thr-144. The hydrophobic residues Leu-255 (alpha1-alpha6 subunits) or Met-255 (alpha7-alpha8 10 subunits) present in MSR II, are replaced by the polar amino acid Gln-255 in the alpha9 subunit. In addition, when compared to other nAChR subunits, alpha9 has a deletion of a Thr residue between MSR II and MSR III.

A full-length alpha9 cDNA suitable for *Xenopus* 15 oocyte expression studies was constructed by subcloning the fragment from nucleotide -94 to 1766 (Figure 1; i.e., residues 79 to 1938 as presented in SEQ ID NO:1) into the expression vector pGEMHE (Liman et al., *Neuron*, 9, 861-871, 1992). cRNA was synthesized using the mMessage mMACHINE 20 transcription kit (Ambion, Austin, TX), with plasmid linearized with *Nhe*I.

Two days after the injection of alpha9 cRNA, more than 95% of voltage-clamped *Xenopus* oocytes responded to acetylcholine. Inward currents in response to 100 μ M acetylcholine ranged from 20 to 500 nA. Figure 4A shows 25 representative current traces in response to the application of acetylcholine. High concentrations ($>10 \mu$ M) of this agonist evoked a fast peak response which rapidly decayed to a plateau level. Oocytes expressing alpha9 were 30 insensitive to glutamate, GABA, glycine, serotonin, ATP, histamine and adenosine.

All functional nAChR alpha subunits cloned prior to the cloning of the alpha9 subunit, upon expression in *Xenopus* oocytes, form either heteromeric or homomeric

receptor-channel complexes activated by nicotine (Boulter et al., *Proc. Natl. Acad. Sci. USA*, 84, 7763-7767, 1987; Duvoisin et al., *Neuron*, 3, 487-496, 1989; Couturier et al., *Neuron*, 5, 847-856, 1990; Luetje and Patrick, *J. 5 Neurosci.* 11, 837-845, 1991; Seguela et al., *J. Neurosci.*, 13, 596-604, 1993; Gerzanich et al., *Molec. Pharmacol.*, 45, 212-220, 1994). Strikingly, nicotine (0.1 μ M to 1 mM) did not elicit any response in alpha9-injected oocytes (Figure 4A). Co-expression of alpha9 with either beta2 or beta4 10 nAChR subunits did not result in the formation of receptor-channels that were activated by nicotine. The alpha9 receptor-channel complex was also not activated by muscarine (Figure 4A). Moreover, neither the nicotinic agonist cytosine nor the muscarinic agonists bethaneol and 15 pilocarpine elicited current responses. However, both the nicotinic agonist 1,1-dimethyl-4-phenylpiperazinium (DMPP) and the muscarinic M1 agonist oxotremorine M (OXO-M), induced inward currents in alpha9-injected oocytes (Figure 4A). Figure 4B shows the concentration-response curves to 20 these cholinergic agonists. Acetylcholine had an apparent affinity (EC_{50}) of 10 μ M. The maximum current responses elicited by both DMPP and OXO-M were approximately 5% of that observed with acetylcholine.

Although neither nicotine nor muscarine evoked 25 responses in oocytes injected with alpha9 cRNA (see Figure 4A), both of these classic cholinergic agonists reduced the currents evoked by acetylcholine. Figure 5A shows the inhibition curves that resulted from the co-application of 30 10 μ M acetylcholine with increasing concentrations of either nicotine or muscarine (IC_{50} = 30 μ M and 75 μ M, respectively). As shown in Figure 5B, the alpha9 receptor-channel complex was also blocked by the nicotinic antagonist d-tubocurarine (IC_{50} = 0.3 μ M), as well as by the 35 muscarinic antagonist atropine (IC_{50} = 1.3 μ M). The alkaloid strychnine, classically used as a blocker of glycine-gated chloride channels, was found to be a potent antagonist of

alpha9 homomers, with an IC_{50} of 0.02 μ M (Figure 5B). Both α -bungarotoxin (100 nM) and κ -bungarotoxin (100 nM) blocked responses to 100 μ M acetylcholine (Figure 6). The blockade by these toxins was almost completely reversed after a 10 5 minute wash of the oocytes with frog Ringer's solution.

Electrophysiological properties were determined on alpha9 injected oocytes 2-7 days after injection. The current-voltage (I-V) relationship obtained by the application of a 2 second voltage ramp at the plateau 10 response to acetylcholine is shown in Figure 7A. The I-V curve was non-linear, displaying a maximal inward current elicited by acetylcholine at -50 mV. Current responses were reduced at potentials negative to -50 mV. The fact that the ratio between the inward current elicited by 100 15 μ M acetylcholine and that evoked by 1 μ M acetylcholine was greater at -50 mV (2.1) than at -80 mV (1.0), indicates that the reduction in current responses at hyperpolarized potentials may depend upon agonist concentration. At holding potentials more positive than -50 mV, the inward 20 currents activated by acetylcholine decreased until -25 mV, where a strong rectification was observed up to a holding potential of +20 mV. I-V curves for both peak and plateau responses performed with stepwise increments in the holding potential, had the same shape as shown in Figure 7A.

25 From the I-V relationships, an apparent reversal potential of -25 mV is estimated. This value is compatible with either a non-selective cationic current or with an anionic (Cl^-) current. The change in external NaCl concentration from 50 mM to 150 mM produced a positive 30 shift in the reversal potential of acetylcholine-induced currents. This indicates that the alpha9 channel is permeable to Na^+ . Most of the peak response elicited by 100 mM acetylcholine in alpha9-expressing oocytes disappeared when oocytes were injected with the calcium chelator 35 1,2-bis (2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid

(BAPTA) (see Figure 7B). Thus, as has been suggested for other nAChR subunits (Gerzanich et al., *Molec. Pharmacol.*, **45**, 212-220, 1994), this result indicates that part of the current evoked by acetylcholine is carried by a Cl⁻ current 5 through Ca²⁺-activated Cl⁻ channels known to be present in oocytes (Miledi and Parker, *J. Physiol. (Lond)*., **357**, 173-183, 1984). In order to further test the participation of a Ca²⁺ activated Cl⁻ current in response to acetylcholine, the reversal potentials of Cl⁻ and Na⁺ were shifted in 10 opposite directions by transiently raising the external NaCl concentration to 350 mM and holding the oocytes at -10 mV under two electrode voltage clamp. Under this condition, 100 μ M acetylcholine elicited both an outward current followed by an inward current (Figure 7C). As 15 reported for other neuronal nAChRs (Vernino et al., *Neuron*, **8**, 127-134, 1992; Seguela et al., *J. Neurosci.*, **12**, 596-604, 1993), the inward current probably results from the influx of cations through alpha9 receptor-channels and the outward current from the flux of Cl⁻ through 20 Ca²⁺-activated Cl⁻ channels. It should be noted that I-V curves performed in 1,2-bis(2-aminophenoxy)ethane N,N,N',N'-tetraacetic acid injected oocytes had the same shape as that described above, suggesting that the Cl⁻ current did not contribute to the I/V curve under the 25 conditions of the experiment.

The above-described *Xenopus* oocyte expression studies demonstrate that the alpha9 protein subunit forms ion channels activated by acetylcholine and permeable to both Na⁺ and Ca²⁺. Similar to the alpha7 and alpha8 neuronal 30 subunits (Couturier et al., *Neuron*, **5**, 847-856, 1990; Gerzanich et al., *Molec. Pharmacol.*, **45**, 212-220, 1994), alpha9 can assemble into a homomeric receptor-channel complex. This differs from other functional neuronal nAChR 35 alpha subunits which require co-assembling with a beta subunit in order to form receptor-channel complexes (Boulter et al., *Proc. Natl. Acad. Sci. USA*, **84**, 7763-7767,

1987; Ballivet et al., *Neuron*, 1, 847-852, 1988; Wada et al., *Science*, 240, 330-334, 1988).

5 Currents elicited by acetylcholine in alpha9-injected oocytes decreased at holding potentials negative to -50 mV. This could result from a voltage-dependent blockade of the channel either by acetylcholine or by cations present in the solution used to maintain the oocytes. The fact that the block was more pronounced at high agonist concentrations indicates that at 10 least part of this effect is due to a voltage-dependent channel block by acetylcholine. High concentrations of acetylcholine and carbamylcholine are known to produce a voltage- and concentration-dependent channel block of muscle nAChR present in BC₃H-1 cells (Sine and Steinbach, 15 *Biophys. J.*, 46, 277-284, 1984).

Based on its primary structure and electrophysiological properties, the alpha9 protein belongs to the nicotinic family of ligand gated ion channels which includes subunits for nAChR, GABA_A, glycine and 5-HT₃ receptors. However, as described earlier, in alpha9-injected oocytes, nicotine, muscarine, d-tubocurarine and atropine blocked acetylcholine-evoked current responses. Therefore, the alpha9 receptor-channel complex falls into neither the nicotinic nor the muscarinic 25 subdivisions of the pharmacological classification scheme of cholinergic receptors (P. Taylor in *The pharmacological basis of therapeutics*, A. Goodman-Gilman, T.H. Rall, A.S. Nies and P. Taylor, eds. (New York:Pergamon Press), pp. 122-149 and 166-186, 1990). The finding that both the 30 nicotinic agonist DMPP and the muscarinic agonist OXO-M are capable of eliciting current responses in alpha9-injected oocytes indicates that the alpha9 receptor exhibits a mixed nicotinic-muscarinic pharmacology. In addition, the 35 blockage of alpha9 receptors by the glycine receptor antagonist strychnine is unusual. A similar effect of

strychnine has also been reported on alpha7 and alpha8 homomers expressed in *Xenopus* oocytes (Seguela et al., *J. Neurosci.*, 12, 596-604, 1993; Gerzanich et al., *Molec. Pharmacol.*, 45, 212-220, 1994).

5 The alpha9 protein subunit contains the most conserved amino acid residues within the proposed acetylcholine binding site of nAChR alpha subunits (Dennis et al., *Biochem.*, 27, 2346-2357, 1988; Galzi et al., *J. Biol. Chem.*, 265, 10430-10437, 1990). Nevertheless, two
10 non-conservative substitutions in the alpha9 protein, Phe-99 to Ser and Lys-144 to Thr (position numbers refer to the mature protein, absent the 28 residues of the leader sequence), are near the first and second domains of the postulated agonist binding site for nAChR. These amino
15 acid substitutions are likely to be responsible for the distinct pharmacological properties of the alpha9 receptor-channel complex.

To determine the tissue expression pattern of the alpha9 gene, *in situ* hybridization studies were performed.
20 In vitro synthesized RNA derived from coding sequence of the alpha9 genomic clone was hybridized to sagittal sections of rat embryos and coronal sections of adult rat brains. The presence of transcripts are observed in the hypophyseal gland of a rat embryo at stage E16 (see Figures
25 9B and 9D). The alpha9 gene expression is observed to be restricted to the pars tuberalis of the adenohypophysis, whereas the pars distalis and the neurohypophysis show no detectable signal. The alpha9 mRNA is also observed to be present in the adult rat pars tuberalis, at the ventral
30 surface of the median eminence (see Figure 9F). Alpha9 expression is also observed throughout the E16 rat olfactory mucosa (see Figure 9B). The alpha9 transcripts are detected in the pseudostratified columnar epithelium that lines each of the turbinates of the olfactory organ.
35 Additional expression is seen in the tongue of the

developing rat (Figure 9B). Finally, *in situ* hybridization analysis performed on 20 mm coronal sections every 180 mm throughout the adult brain, did not establish alpha9 gene expression in the central nervous system of the rat.

5 *In situ* hybridization studies performed on cryostat sections of the rat cochlea indicate that the alpha9 gene is also expressed in the outer hair cell region of all cochlea turns. No expression of the alpha9 gene was observed in spiral ganglion neurons or other supporting
10 structures of the cochlea (see Figure 9B).

Previously published neuronal nAChR genes are reported as being expressed in the central nervous system of vertebrates (Sargent, *Annu. Rev. Neurosci.*, 16, 403-443, 1993). As disclosed above, *in situ* hybridization studies
15 performed in coronal sections throughout the rat brain did not establish alpha9 gene expression in the central nervous system. Although low levels of alpha9 transcripts or a very restricted expression pattern which escaped detection cannot be ruled out, the results suggest that relative to
20 other nAChR subunits, alpha9 may be involved in a distinct subset of cholinergic functions *in vivo*. *In situ* hybridization studies showed that in the rat the alpha9 subunit gene is expressed in the pars tuberalis of the pituitary, the olfactory epithelium, the outer hair cells
25 of the cochlea and the skeletal muscle of the tongue.

The pars tuberalis constitutes an anatomically well defined part of the vertebrate adenohypophysis consisting of peptide-secreting cells, gonadotropes and thyrotropes (Wittkowski et al., *Acta Endocrinol.*, 126, 30 285-290, 1992). Neuroendocrine effects, such as the inhibition of luteinizing hormone and thyroid-stimulating hormone secretion in response to nicotine exposure, has been reported in humans and rats (Fuxe et al., *Psychoneuroendocrinol.*, 14, 19-41, 1989). Although these

effects have been attributed to the activation of hypothalamic nAChR, the presence of the alpha9 nAChR subunit in the pituitary indicates that nicotine may exert a direct action on this gland.

5 It is likely that the olfactory sensory cells receive efferent innervation that modulates olfactory function (Shirley, *Olfaction. Intl. Rev. Neurobiol.*, 33, 1-53, 1992). A cholinergic modulation has been suggested, since the application of acetylcholine causes slow
10 electrical potentials and modifies spike activity in olfactory receptor neurons (Bouvet et al., *Neurosci. Res.*, 5, 214-223, 1988). Although further pharmacological characterization of the acetylcholine response in olfactory neurons as well as a more precise localization of the
15 alpha9 subunit within the olfactory epithelium are necessary, the presence of alpha9 transcripts in the olfactory epithelium could provide the molecular basis for the cholinergic effect described.

The alpha9 gene expression in the developing
20 muscle of the tongue is intriguing. With the *in situ* hybridization studies performed it is not possible to distinguish whether the signal is actually localized in muscle fibers or in the surrounding connective tissue. However, alpha9 transcripts appear not to be present in all
25 developing skeletal muscles. For example, *in situ* hybridization studies performed in mid-sagittal sections of rat embryos showed no evidence of alpha9 transcripts in the intercostal or axial muscles.

The overall pharmacological characteristics of
30 the homomeric alpha9 receptor expressed in oocytes differ from those of other cloned nAChRs (Boulter, et al., *Proc. Natl. Acad. Sci. USA*, 84, 7763-7767, 1987; Ballivet, et al., *Neuron*, 1, 847-852, 1988; Wada, et al., *Science*, 240,

330-334, 1988; Couturier, et al., *Neuron*, 5, 847-856, 1990; Gerzanich, et al., *Molec. Pharmacol.*, 45, 212-220, 1994).

In order to further investigate the expression pattern of the alpha9 gene in the rat cochlea, PCR was 5 performed on cDNA reverse transcribed from cochlear total RNA. Two primers specific for the alpha9 sequence were designed and used to amplify a fragment that spans an intron-exon boundary of the alpha9 gene. As shown on Figure 8, a fragment of the expected size (573 bp) was 10 amplified from rat cochlear cDNA with alpha9 primers. Restriction endonuclease analysis of the fragment with *Acc*I, *Hinf*I and *Nco*I, further confirmed that it was derived from alpha9 transcripts. Since the alpha9 gene is also transcribed in the rat olfactory epithelium, RNA obtained 15 from this tissue was used as a positive control. Rat sciatic nerve cDNA was included as a negative control to rule out the possibility that, with the parameters used for the PCR, very low levels of transcripts would be detected in any tissue studied. Whereas no DNA was amplified from 20 the sciatic nerve using specific primers for alpha9 (see Figure 8), both alpha3 and alpha4 subunits could be detected in this tissue with their respective specific primers.

A possible physiological role for the alpha9 25 receptor-channel is the efferent cholinergic innervation of cochlea hair cells. Outer hair cells of the cochlea are implicated in the mechanical amplification of sound in vertebrates (Flock, R. Klinke and R. Hartmann, eds. (Berlin:Springer-Verlag), pp. 2-8, 1983). These cells 30 receive efferent cholinergic innervation. The electrical stimulation of these efferent neurons results in reduction of sensitivity and tuning of the auditory nerve fibers, which in turn could induce protection against acoustic trauma (Brown and Nuttal, *J. Physiol. (Lond.)*, 354, 35 625-646, 1984; Klinke, *Hearing Res.*, 22, 235-243, 1986;

Rajan and Johnstone, *Brain Res.*, 458, 241-255, 1988). The molecular nature of the acetylcholine receptor involved in the efferent innervation of cochlear hair cells has not been described. Although both a non-selective cation channel as well as a G-protein coupled receptor have been proposed, cholinergic agonists and antagonists have been of little benefit to characterize this receptor as either nicotinic or muscarinic (Housley and Ashmore, *Proc. R. Soc. Lond. B*, 244, 161-167, 1991; Fuchs and Murrow, *Proc. R. Soc. Lond. B*, 248, 35-40, 1992; Fuchs and Murrow, *J. Neurosci.*, 12, 800-809, 1992; Kakehata et al., *J. Physiol. (Lond.)*, 463-, 227-244, 1993; Erostegui et al., *Hearing Res.*, 74, 135-147, 1994). Therefore, whatever the primary structure for this cholinergic receptor might be, based on its unique pharmacological characteristics it has been suggested that it is of a receptor type not previously described (Fuchs and Murrow, *Proc. R. Soc. Lond. B*, 248, 35-40, 1992; Erostegui et al., *Hearing Res.*, 74, 135-147, 1994).

The results presented herein suggest that the alpha9 receptor is the cholinergic component of the cochlear efferent system. This conclusion is based primarily on the presence of alpha9 transcripts in the hair cells of the rat cochlea. Evidence to date suggests that the cochlear efferent system is involved in improving the detection of signal within background noise, protection of the cochlea from noise damage, and attenuating the cochlear response to auditory stimulation when attention must be focused elsewhere.

Various experiments have shown that the cholinergic component of the cochlear efferent system may also be involved in aminoglycoside antibiotic ototoxicity. When administered in high doses, these antibiotics cause outer hair cells (OHC) to degenerate (Govaerts, et al., *Toxicology Letters*, 52, 227-251, 1990). The results of

such degeneration ranges from ringing in the ears to total loss of hearing. Current theories regarding the mechanisms whereby aminoglycosides exert their ototoxic effect upon the OHCs suggest that the OHCs become metabolically destabilized due to a block of intracellular messaging systems. At the same time, the efferent synapses are also destabilized, and can no longer monitor and control the amount of ACh released following stimulation. The end result is that there is an overstimulation (an excess of ACh) directed toward the destabilized OHCs, which results in the degeneration observed (Williams, et al., *Hearing Res.*, 30, 11-18, 1987). Thus, ACh, and the alpha₉ receptor responsible for transducing the efferent signal from the efferent terminal to the hair cell, are intimately involved in releasing the ototoxic potential of the aminoglycoside antibiotics. Accordingly, antagonists to receptors comprising at least one alpha₉ receptor subunit (i.e., alpha₉ blockers) will reduce or eliminate the side-effects of aminoglycoside-induced ototoxicity.

The present invention provides isolated nucleic acids encoding an alpha₉ nicotinic acetylcholine receptor subunit. The term "nucleic acids" (also referred to as polynucleotides) encompasses RNA as well as single and double-stranded DNA and cDNA. As used herein, the phrase "isolated polynucleotide" refers to a polynucleotide that has been separated or removed from its natural environment. One means of isolating a polynucleotide encoding an alpha₉ nAChR receptor subunit is to probe a mammalian genomic library with a DNA probe using methods well known in the art. DNA probes derived from the alpha₉ receptor gene particularly useful for this purpose. DNA and cDNA molecules that encode alpha₉ receptors can be used to obtain complementary genomic DNA, cDNA or RNA from human, mammalian, or other animal sources. Such molecules can also be used to isolate related cDNA or genomic clones by screening cDNA or genomic libraries, by methods described

in more detail below. Invention nucleic acids includ
nucleotid sequences that are substantially the same as the
nucleotide sequence shown in Figure 1 (see also SEQ ID
NO:1). The present invention also encompasses nucleic
5 acids which are degenerate variants of the nucleotide
sequence shown in Figure 1 (and SEQ ID NO:1).

The term "degenerate variants" refers to nucleic
acids encoding alpha9 nAChR subunits that, by virtue of the
degeneracy of the genetic code, do not necessarily
10 hybridize to the invention nucleic acids under specified
hybridization conditions. Preferred nucleic acids encoding
invention polypeptide(s) or proteins(s) are comprised of
nucleotides that encode substantially the same amino acid
sequence set forth in Figure 1 (see also SEQ ID NO:2).
15 Alternatively, preferred nucleic acids encoding invention
polypeptide(s) hybridize under high stringency conditions
to substantially the entire sequence, or substantial
portions (i.e., typically at least 25-30 contiguous
nucleotides) of the nucleotide sequence set forth in Figure
20 1 (see also SEQ ID NO:1).

Stringency of hybridization, as used herein,
refers to conditions under which polynucleotide hybrids are
stable. As known to those of skill in the art, the
stability of hybrids is a function of sodium ion
25 concentration and temperature. (See, for example, Sambrook
et al., Molecular Cloning: A Laboratory Manual 2d Ed. (Cold
Spring Harbor Laboratory, 1989; incorporated herein by
reference).

The invention provides isolated alpha9 nicotinic
30 acetylcholine receptor subunit peptide, polypeptide and/or
protein encoded by invention nucleic acids and alpha9
nicotinic acetylcholine receptor comprising said subunit.
The alpha9 nAChR subunit comprises a protein of
approximately 451 amino acids in length. The amino acid

sequence of the alpha9 subunit is set forth in Figure 1 (and in SEQ ID NO:2).

As used herein, the term "isolated protein" refers to a protein free of cellular components and/or 5 contaminants normally associated with a protein in its native *in vivo* environment. Invention polypeptides and/or proteins include naturally occurring allelic variants, as well as recombinant forms thereof. The alpha9 nAChR polypeptide can be isolated using various methods well 10 known to those of skill in the art. The methods available for the isolation and purification of invention proteins include, precipitation, gel filtration, ion-exchange, reverse-phase and affinity chromatography. Other well-known methods are described in Deutscher et al., Guide 15 to Protein Purification: Methods in Enzymology Vol. 182, (Academic Press, 1990), which is incorporated herein by reference. Alternatively, isolated polypeptides of the present invention can be obtained using well-known recombinant methods as described, for example, in Sambrook 20 et al., Molecular Cloning: A Laboratory Manual 2d Ed. (Cold Spring Harbor Laboratory, 1989; incorporated herein by reference).

Invention polypeptide(s) can be produced by expressing nucleic acids encoding the alpha9 nAChR subunit 25 in a suitable host cell, such as a bacterial cell, a yeast cell, an amphibian cell (i.e., oocyte), or a mammalian cell, using methods well known in the art. The expressed polypeptide can be recovered using well-known methods. Invention polypeptides can be isolated directly from cells 30 that have been transformed with expression vectors, described below in more detail. The invention polypeptide, biologically active fragments, and functional equivalents thereof can also be produced by chemical synthesis. As used herein, "biologically active fragment" refers to any 35 portion of the alpha9 polypeptide represented by the amino

acid sequence in Figure 1 (see also SEQ ID NO:2) that can assemble into a cationic channel activated by acetylcholine and permeable to calcium. Synthetic polypeptides can be produced, for example, using Applied Biosystems, Inc. Model 5 430A or 431A automatic peptide synthesizer (Foster City, CA) employing the chemistry provided by the manufacturer.

As used herein, the phrase "nicotinic acetylcholine receptor (nAChR) subunit" refers to recombinantly expressed/produced (i.e., isolated or 10 substantially pure) protein that contains four highly hydrophobic regions which predict membrane spanning regions and cysteine residues at positions 127, 141, 191 and 192 (referring to the mature peptide, not including the 28 amino acid leader sequence). Such protein subunits 15 assemble into a cationic channel which is activated by acetylcholine. Invention nAChR subunits include variants thereof encoded by mRNA generated by alternative splicing of a primary transcript, as well as biologically active fragments.

20 The alpha9 nAChR subunit of the invention contributes to the formation of a functional receptor, as assessed by methods described herein, by combining with at least one additional nAChR subunit of the same or different type. As used herein, the phrase "functional receptor" 25 means that the binding of a ligand, for example, acetylcholine (ACh), causes the receptor ion channel to open thereby permitting cations, such as Ca^{2+} , as well as Na^+ and K^+ , to enter the cell. Agonist activation of a "functional invention receptor" induces the receptor.

30 Modification of invention nucleic acids, polypeptides or proteins with the following phrases: "recombinantly expressed/produced", "isolated", or "substantially pure", encompasses nucleic acids, peptides, polypeptides or proteins that have been produced in such

form by the hand of man, and are thus separated from their native *in vivo* cellular environment. As a result of this human intervention, the recombinant nucleic acids, polypeptides and proteins of the invention are useful in 5 ways that the corresponding naturally occurring molecules are not, such as identification of compounds as potential drugs.

Sequences having "substantial sequence homology" are intended to refer to nucleotide sequences that share at 10 least about 90% identity with invention nucleic acids; and amino acid sequences that typically share at least about 95% amino acid identity with invention polypeptides. It is recognized, however, that polypeptides or nucleic acids containing less than the above-described levels of homology 15 arising as splice variants or generated by conservative amino acid substitutions, or by substitution of degenerate codons are also encompassed within the scope of the present invention.

The present invention also provides nucleic acids 20 encoding alpha9 receptor subunit(s) operatively linked to a promoter, as well as other regulatory sequences. As used herein, the phrase "operatively linked" refers to the functional relationship of the nucleic acid with regulatory and effector sequences, such as promoters, enhancers, 25 transcriptional and translational stop sites, and other signal sequences. Specifically operative linkage of a nucleic acid to a promoter refers to the physical and functional relationship between the nucleic acid and the promoter such that transcription of DNA is initiated from 30 the promoter by an RNA polymerase that specifically recognizes and binds to the promoter.

Suitabl promoters include specific sequences that are sufficient for RNA polymeras recognition, binding and transcription initiation. Additionally, suitable

promoters include sequences that modulate the recognition, binding and transcription initiation activity of RNA polymerase. Such sequences may be *cis* acting or may be responsive to *trans* acting factors. Depending upon the 5 nature of the regulation, promoters may be constitutive or regulated. Examples of promoters are SP6, T4, T7, SV40 early promoter, cytomegalovirus (CMV) promoter, mouse mammary tumor virus (MMTV) steroid-inducible promoter, Moloney murine leukemia virus (MMLV) promoter, and the 10 like.

Vectors employed in the present invention contain both a promoter and a cloning site into which nucleic acid encoding alpha9 receptor subunit(s) can be operatively linked. Such vectors, which are well known in the art, are 15 capable of transcribing RNA *in vitro* or *in vivo*, and are commercially available from sources such as Stratagene (La Jolla, CA) and Promega Biotech (Madison, WI). In order to optimize expression and/or *in vitro* transcription, it may be necessary to remove, add or alter 5' and/or 3' 20 untranslated portions of the clones to eliminate extra, potentially inappropriate alternative translation initiation codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus 25 ribosome binding sites can be inserted immediately 5' of the start codon to enhance expression. (See, for example, Kozak, *J. Biol. Chem.* 266:19867 (1991)). Similarly, alternative codons, encoding the same amino acid, can be substituted for native codons of the alpha9 nAChR subunit 30 in order to enhance transcription (e.g., the codon preference of the host cell can be adopted, the presence of G-C rich domains can be reduced, and the like).

Examples of suitable vectors that may be employed in the present invention include viruses, such as 35 baculoviruses and retroviruses, bacteriophages, cosmids,

plasmids and other recombination vehicles typically used in the art. Invention nucleic acids are inserted into vector genomes using methods well known in the art. For example, insert and vector DNA can be contacted, under suitable 5 conditions, with a restriction enzyme to create complementary ends on each molecule that can pair with each other and be joined together with a ligase. Alternatively, synthetic linkers can be ligated to the termini of restricted invention nucleic acids. These synthetic 10 linkers contain nucleic acid sequences that correspond to a particular restriction site in the vector DNA. Additionally, a nucleic acid containing a termination codon and an appropriate restriction site can be ligated into a vector containing, for example, some or all of the 15 following: a selectable marker gene, such as the neomycin gene for selection of stable or transient transfected cells in mammalian cells; enhancer/promoter sequences from the immediate early gene of human CMV for high levels of transcription; transcription termination and RNA processing 20 signals from SV40 for mRNA stability; SV40 polyoma origins of replication and ColE1 for proper episomal replication; versatile multiple cloning sites; and T7 and SP6 RNA promoters for *in vitro* transcription of sense and antisense RNA. Other means are well known and available in the art.

25 Also provided are vectors comprising nucleic acid encoding alpha9 nAChR subunit, which vectors are adapted for expression in a bacterial cell, a yeast cell, an amphibian cell (i.e., oocyte), a mammalian cell or other animal cells. Such vectors additionally comprise 30 regulatory elements necessary for expression of nucleic acid in the bacterial, yeast, amphibian, mammalian or animal cells located relative to the nucleic acid encoding alpha9 nAChR subunit so as to permit expression thereof. As used herein, "expression" refers to the process by which 35 nucleic acids are transcribed into mRNA and translated into peptides, polypeptides, or proteins. If the nucleic acid

is derived from genomic DNA, expression may include splicing of the mRNA, if an appropriate eucaryotic host is selected. Regulatory elements required for expression include promoter sequences to bind RNA polymerase and 5 transcription initiation sequences for ribosome binding. For example, a bacterial expression vector might include a promoter such as the lac promoter, the Shine-Dalgarno transcription initiation sequence and the start codon AUG (Sambrook et al., Molecular Cloning: A Laboratory Manual 2d 10 Ed. (Cold Spring Harbor Laboratory, 1989; incorporated herein by reference) Similarly, a eucaryotic expression vector might include a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment 15 of the ribosome. Such vectors can be obtained commercially or assembled from available sequences and by methods well known in the art.

This invention also provides a transformed host that expresses recombinant alpha9 nicotinic acetylcholine 20 receptor. Such a host has been transformed with a nucleic acid encoding alpha9 nAChR subunit. An example of a transformed host according to the present invention is a mammalian cell comprising a plasmid specifically adapted for expression in such a cell. The plasmid contains a 25 nucleic acid encoding an alpha9 nAChR subunit and the regulatory elements necessary for expression of the subunit. Suitable mammalian cells that may be utilized in the present invention include, for example, mouse fibroblast NIH3T3 cells, CHO cells, HeLa cells, Ltk⁻ cells, 30 PC12 and N2A neuronal cells, HEK-293 kidney cells and CG4 glial cells. Host cells may be transformed with plasmids such as those described *supra* by methods well known in the art such as calcium phosphate precipitation, DEAE-dextran, electroporation, microinjection or lipofection. Other 35 suitable hosts that may be employed in the present invention are oocytes, particularly *Xenopus* oocytes.

Nicotinic acetylcholine receptors, according to the invention, are recombinantly expressed in a host cell containing at least one alpha9 subunit. Recombinant receptors may be homomeric or heteromeric. Thus, a 5 transformed host cell can recombinantly express a receptor containing only alpha9 subunits, or containing at least one alpha9 subunit and one or more other nAChR subunits.

The present invention also provides nucleic acid probes. Such probes comprise a polynucleotide capable of 10 specifically hybridizing with a sequence encoding an alpha9 nAChR subunit. As used herein, the term "probe" refers to single-stranded or double-stranded DNA or RNA that has a sequence of nucleotides that includes at least 14 contiguous bases set forth in Figure 1 (see also SEQ ID 15 NO:1). Probes used to distinguish the alpha9 subunit from other alpha nAChRs subunits will preferably consist of at least 14 contiguous bases from the cytoplasmic loop region of the alpha9 nucleotide sequence. Alternatively, probes that are to be used to find additional subunits of the 20 nAChR family will preferably consist of at least 14 contiguous bases from a membrane spanning region of the alpha9 nucleotide sequence.

As used herein, the phrase "specifically hybridizing" encompasses the ability of a polynucleotide to 25 recognize a nucleic acid sequence that is complementary thereto and to form double-helical segments via hydrogen bonding between complementary base pairs. Nucleic acid probe technology is well known to those skilled in the art who will readily appreciate that such probes may vary 30 greatly in length and may be labeled with a detectable agent, such as a radioisotope, a fluorescent dye, and the like, to facilitate detection of the probe. Invention probes are useful to detect the presence of nucleic acids encoding the alpha9 nAChR subunit. For example, the probes 35 can be used for *in situ* hybridizations to identify specific

tissues in which the alpha9 nAChR subunit gene is expressed. Additionally, oligonucleotides complementary to nucleic acids encoding the alpha9 nAChR subunit are useful for detecting the alpha9 gene and associated mRNA, or for 5 the isolation of related genes using homology screening of genomic or cDNA libraries, or by using amplification techniques well known to those of skill in the art.

The invention further provides antisense 10 oligonucleotides having a sequence capable of binding specifically with any portion of an mRNA that encodes the alpha9 nAChR subunit so as to prevent translation of the mRNA. Antisense oligonucleotides may also contain a sequence capable of binding specifically with any portion 15 of the cDNA encoding the alpha9 subunit. As used herein, the phrase "binding specifically" encompasses the ability of a nucleic acid sequence to recognize a complementary nucleic acid sequence and to form double-helical segments therewith via the formation of hydrogen bonds between the 20 complementary base pairs.

Also provided by the present invention are compositions comprising an amount of an invention antisense oligonucleotide effective to reduce expression of the alpha9 nAChR subunit wherein said antisense oligonucleotide 25 is capable of binding with mRNA encoding the alpha9 nAChR receptor so as to prevent its translation. Compositions provided by the present invention comprise an acceptable hydrophobic carrier capable of passing through cell membranes and may also comprise a structure which binds to 30 a receptor specific for a selected cell type and is thereby taken up by cells of the selected cell type. The structure may be part of a protein known to bind to a cell-type specific receptor.

Antisense oligonucleotide compositions (AOCs) 35 according to the present invention are designed to be

stable in the blood stream for administration to a subject by injection, or in laboratory cell culture conditions. The physical and chemical properties of the AOC are selected so that the composition is capable of passing 5 through the cell membrane in order to enter the cell cytoplasm. Such a composition can be designed to include small, hydrophobic chemical structures, or alternatively, specific cell transport systems which facilitate and transport the AOC into the cell. In addition, the AOC can 10 be designed for administration only to certain selected cell populations by targeting the AOC to be bound and taken up by select cell populations. Targeting can be accomplished by designing cell specific AOCs to bind to a receptor found only in a certain cell type, as discussed 15 *supra*. Alternatively, an AOC can also be designed to recognize and selectively bind to a target mRNA sequence. In the latter instance, targeting is accomplished, for example, by employing a sequence contained within the sequence shown in Figure 1 (SEQ ID NO:1). The AOC is 20 designed to inactivate the target mRNA sequence by (1) binding to target mRNA and inducing degradation of the mRNA by, for example, RNase I digestion, or (2) inhibiting translation of target mRNA by interfering with the binding of translation-regulating factors or ribosomes, or by 25 inclusion of other chemical structures, such as ribozyme sequences or reactive chemical groups which either degrade or chemically modify the target mRNA. AOCs have been shown to be capable of such properties when directed against mRNA targets (see Cohen et al., *TIPS*, 10:435 (1989) and 30 Weintraub, *Sci. American*, January (1990), pp. 40; both incorporated herein by reference).

The invention also provides antibodies having specific reactivity with alpha9 nAChR polypeptides and/or proteins of the subject invention. Active fragments of 35 antibodies are encompassed within the definition of "antibody".

The antibodies of the invention can be produced by methods known in the art. For example, polyclonal and monoclonal antibodies can be produced by methods described, for example, in Harlow and Lane, *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory 1988), which is incorporated herein by reference. The alpha9 protein of the invention, or portions thereof, can be used as the immunogen in generating such antibodies. Alternatively, synthetic peptides can be prepared (using commercially available synthesizers) and used as immunogens. Amino acid sequences can be analyzed by methods well known in the art to determine whether they encode hydrophobic or hydrophilic domains of the corresponding alpha9 invention protein. Altered antibodies such as chimeric, humanized, CDR-grafted or bifunctional antibodies can also be produced by methods well known in the art. Such antibodies can also be produced by hybridoma, chemical synthesis or recombinant methods described, for example, in (Sambrook et al., *Molecular Cloning: A Laboratory Manual* 2d Ed. (Cold Spring Harbor Laboratory, 1989); incorporated herein by reference and Harlow and Lane, *supra*). Both anti-peptide and anti-fusion protein antibodies can be used. (see, for example, Bahouth et al., *Trends Pharmacol. Sci.* 12:338 (1991); Ausubel et al., *Current Protocols in Molecular Biology* (John Wiley and Sons, NY 1989) which are incorporated herein by reference).

The invention antibodies have various uses, such as, for example, isolation of the alpha9 invention receptor. Additionally, the antibodies are useful for detecting the presence of the alpha9 receptor, as well as analysis of receptor localization, subunit composition, and structure of functional domains. A method for detecting the presence of alpha9 nAChRs on the surface of a cell comprises contacting the cell with an antibody that specifically binds alpha9 nACh receptor and detecting the presence of the bound antibody on the cell surface. With

respect to the detection of alpha9 receptors, the invention antibodies can be used, for example, for in vitro diagnostic or in vivo imaging methods.

Immunological procedures useful for in vitro detection of alpha9 receptor in a sample include immunoassays that employ a detectable antibody. Such immunoassays include, for example, ELISA, Pandex microfluorimetric assay, agglutination assays, flow cytometry, serum diagnostic assays and immunohistochemical staining procedures which are well known in the art. An antibody can be made detectable by various means well known in the art. For example, a detectable marker can be directly or indirectly attached to the antibody. Useful markers include, for example, radionuclides, enzymes, fluorogens, chromogens and chemiluminescent labels.

Further, invention antibodies can be used to modulate the ion channel activity of the alpha9 receptor in animals and humans as well as biological tissues isolated therefrom. Accordingly, the invention provides compositions comprising a carrier and an amount of an antibody having specificity for the alpha9 receptor effective to block binding of naturally occurring ligands to the receptor. A monoclonal antibody directed to an epitope of an alpha9 receptor present on the surface of a cell wherein said antibody has an amino acid sequence substantially the same as an amino acid sequence set forth in Sequence ID No. 2 can be useful for this purpose.

The invention further provides a transgenic non-human mammal capable of expressing nucleic acid encoding alpha9 protein. Also provided are transgenic non-human mammals incapable of expressing nucleic acid encoding biologically functional alpha9 protein or alternatively, capable only of expressing alpha9 protein that is biologically deficient in some respect. Varying

degrees of dysfunctionality are achieved through manipulation of alpha9 nucleic acid to encode a mutated protein.

The present invention also provides a transgenic 5 non-human mammal having a genome comprising antisense nucleic acid which is transcribed into antisense mRNA complementary to alpha9 mRNA. Such antisense mRNA hybridizes to alpha9 mRNA and reduces the translation thereof.

10 Nucleic acids employed in transgenic animals of the invention may be associated with an inducible promoter and/or tissue specific regulatory elements, so that expression can be induced, or restricted to specific cell types. Examples of suitable promoters are the 15 metallothionein promoter and the L7 promoter.

The transfer of nucleic acid material into mammalian hosts for the purpose of generating transgenic animals can be accomplished by microinjection, retroviral infection or other means well known to those skilled in the 20 art, of the material into appropriate fertilized embryos. (See, for example, Hogan et al., *Manipulating the Mouse Embryo: A Laboratory Manual* (Cold Spring Harbor Laboratory, 1986). Homologous recombination can also be used for the generation of transgenic animals according to the present 25 invention. Homologous recombination techniques are well known in the art. Homologous recombination replaces a native (endogenous) gene with a recombinant or mutated gene to produce an animal that cannot express a native (endogenous) alpha9 receptor subunit but can express, for 30 example, a mutated receptor subunit. In contrast to homologous recombination, microinjection adds genes to the host genome, without removing host genes. Microinjection can produce a transgenic animal that is capable of expressing both endogenous and exogenous alpha9 receptor

subunits. Transgenic animal model systems are useful for in vivo screening of compounds for identification of receptor-specific ligands, i.e., agonists and antagonists, which activate or inhibit receptor responses.

5 Nucleic acids, oligonucleotides (including antisense), vectors containing same, transformed hosts, receptor subunits and combinations thereof, as well as antibodies of the present invention, can be used to screen compounds *in vitro* to identify those compounds which
10 function as agonists or antagonists of alpha9 receptor subunits of the invention. Such *in vitro* screening assays provide useful information regarding the function and activity of alpha9 receptor subunits of the invention, which can facilitate the identification and design of drugs
15 that are capable of specific interaction with one or more types of receptor subunits or receptor subtypes.

The present invention also provides a method for identifying compounds which bind to alpha9 nicotinic acetylcholine receptor subunits. In such a method, 20 invention receptor subunits may be employed in a competitive binding assay. Such an assay can accommodate the rapid screening of a large number of compounds to determine which compounds, if any, are capable of binding to the alpha9 nAChR subunit. Subsequently, more detailed 25 assays can be carried out with those compounds found to bind, to further determine whether such compounds act as agonists or antagonists of invention receptors (i.e., nAChRs comprising at least one alpha9 subunit).

The present invention still further provides a 30 bioassay for identifying compounds which modulate the activity of receptors of the invention (i.e., nAChRs comprising at least one alpha9 subunit). In one embodiment, the bioassay is conducted by providing cells expressing receptor comprising at least one alpha9 subunit

with at least one potential agonist and thereafter monitoring the cells for changes in ion channel activity. In yet another embodiment, the bioassay is conducted by contacting cells expressing receptor comprising at least 5 one alpha9 subunit with a constant amount of a known alpha9 agonist and increasing amounts of at least one potential antagonist and thereafter monitoring the cells for changes in ion channel activity.

The present invention also provides a bioassay 10 for identifying compounds which modulate the regulatory regions of the alpha9 nAChR subunit gene. Such an assay is conducted utilizing mammalian cells transformed with a nucleic acid construct comprising at least a portion of the regulatory region of the alpha9 gene operatively associated 15 with a reporter gene. The transformed cells are contacted with at least one compound wherein the ability of said compound to modulate the regulatory region is unknown. Thereafter, the cells are monitored for expression of the reporter gene. Suitable reporter genes that may be 20 employed include, for example, the chloramphenicol acetyltransferase gene, the luciferase gene, and the like.

A compound or a signal that "modulates the activity" of an invention receptor refers to a compound or a signal that alters the activity of the alpha9 receptor so 25 that the receptor is different in the presence of the compound or signal than in the absence of the compound or signal. Compounds affecting modulation include agonists and antagonists. An agonist encompasses a compound such as acetylcholine, that activates alpha9 receptor function. 30 Alternatively, an antagonist includes a compound that interferes with alpha9 receptor function. Typically, the effect of an antagonist is observed as a blocking of agonist-induced receptor activation. Antagonists include competitive as well as non-competitive antagonists. A 35 competitive antagonist (or competitive blocker) interacts

with or near the site specific for agonist binding. A non-competitiv antagonist or blocker inactivates the function of the receptor by interacting with a site other than the agonist interaction site.

5 As understood by those of skill in the art, bioassay methods for identifying compounds that modulate nAChR activity generally require comparison to a control. One type of "control" is a cell or culture that is treated substantially the same as the test cell or test culture 10 exposed to the compound, with the distinction that the "control" cell or culture is not exposed to the compound. For example, in methods that use voltage clamp electrophysiological procedures, the same cell can be tested in the presence or absence of compound, by merely 15 changing the external solution bathing the cell. Another type of "control" cell or culture that can be employed is a cell or culture that is identical to transfected cells, with the exception that the "control" cell or culture does not express functional alpha9 nACh receptor subunit. 20 Accordingly, the response of the transfected cell to compound is compared to the response (or lack thereof) of the "control" cell or culture to the same compound under the same reaction conditions.

In still another embodiment of the present 25 invention, the ion channel activity of alpha9 nAChR can be modulated by contacting the receptors with an effective amount of at least one compound identified by any of the above-described bioassays.

30 The following examples are intended to illustrate, but not limit, the present invention.

EXAMPLE I
Screening of Genomic Libraries

A full-length alpha7 nAChR subunit cDNA (Seguela, et al., J. Neurosci., 13, 596-604, 1993) was used to screen 5 5×10^5 clones of a lambdaCharon 4A rat genomic library (obtained from Dr. James Eberwine, Department of Pharmacology, University of Pennsylvania Medical School, Philadelphia, PA). Hybridization was carried out at 65°C in 1 M NaCl, 50 mM Tris-HCl, pH 8.0, 0.5% SDS, 100 mg/ml 10 denatured salmon sperm DNA and 0.1% (w/v) each of Ficoll, polyvinylpyrrolidone and bovine serum albumin. Filters were washed at 45°C in 2 x SSPE (1 x SSPE is 180mM NaCl, 9mM Na₂HPO₄, 0.9mM NaH₂PO₄ and 1mM EDTA, pH 8.0). A clone of 15 ~16kb containing exons IV and V of the alpha9 subunit gene was isolated.

EXAMPLE II
Screening of a cDNA Library

A PCR fragment derived from coding sequences (nucleotides 283 to 806, Figure 1; i.e., nucleotides 455 to 20 979 of SEQ ID NO:1) of the rat genomic clone described in EXAMPLE I was used as a probe to screen 1×10^6 plaques of a lambdaNM1149 adult rat olfactory epithelium cDNA library (obtained from Dr. Heinz Breer, and Dr. Klaus Raming, University Stuttgart-Hohenheim, Institute of Zoophysiology, 25 Stuttgart, Germany). Hybridization was as described in EXAMPLE I and filters were washed at 65°C in 0.2 x SSPE. Four independent clones were isolated, one contained a full-length alpha9 cDNA (Figure 1). The alpha9 cDNA consists of an 87 bp 5' untranslated region, an open 30 reading frame of 1437 bp and 413 bp of 3' untranslated region. The full length alpha9 cDNA was used as a probe to screen two mouse (129SvJ) genomic libraries constructed in phage vectors lambda DASHII and lambda FIXII. Two overlapping genomic clones were obtained (Figure 2). The se

clones, spanning the entire coding sequence of the alpha9 subunit gene were cloned into plasmid vectors and the alpha9 subunit gene structure was determined by sequencing across the intron-exon boundaries.

5

EXAMPLE III

Nucleotide Sequence Determination and Analysis

The alpha9 subunit cDNA clone was sequenced using the Sequenase 2.0 kit (United States Biochemical, Cleveland, OH) and synthetic oligonucleotide primers. A 10 comparison of the alpha9 amino acid sequences with other nAChR alpha subunits was made using sequence analysis software from the University of Wisconsin Genetics Computer Group [Devereux, et al., *Nucl. Acids. Res.*, 12, 387-395, 1984]. The percent sequence identity between paired 15 sequences was calculated by dividing the number of identical residues by the total number of residues in the shorter of the sequences and multiplying the quotient by 100.

20

EXAMPLE IV

Electrophysiological Procedures

A full-length alpha9 cDNA suitable for *Xenopus* oocyte expression studies was constructed by subcloning the fragment from nucleotide -94 to 1766 (Figure 1; i.e., residues 79 to 1938 as presented in SEQ ID NO:1) into the 25 expression vector pGEMHE (Liman, et al., *Neuron*, 9, 861-871, 1992). cRNA was synthesized using the mMessage mMachine transcription kit (Ambion, Austin, TX), with plasmid linearized with *Nhe*I.

The isolation and maintenance of oocytes has been 30 previously described (Boulter, et al., *Proc. Natl. Acad. Sci. USA*, 84, 7763-7767, 1987). Each oocyte was injected with 1 to 10ng of cRNA. Electrophysiological recordings

were performed 2 to 7 days after injection, under two-electrode voltage clamp with an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA). Voltage electrodes were filled with 3M KCl and had a resistance of ~10 M Ω ; 5 current electrodes were filled with 0.3M KCl and had a resistance of ~1 M Ω . Unless otherwise stated, the holding potential was -50 mV. I-V relationships were obtained with pClamp 5.5 software (Axon Instruments), by applying 2 second voltage ramps in the presence of agonist and 10 subtracting the control average values obtained before and after agonist application. All records were digitized and stored on a computer. Data was analyzed using software that was designed and provided by Dr. S. Traynelis (The Salk Institute for Biological Studies, La Jolla, CA).

15 Oocytes were continuously superfused in frog Ringer's solution (10mM HEPES, pH 7.2, 115mM NaCl, 1.8mM CaCl₂ and 2.5mM KCl). No responses were observed by the application of drugs to uninjected oocytes. For the inhibition curves (see Figures 4B, 5A and 5B), antagonists 20 were coapplied with 10 μ M acetylcholine. In the case of α -bungarotoxin and κ -bungarotoxin (see Figures 6A and 6B), oocytes were preincubated with these drugs for 30 minutes. The mean and standard error of the mean of peak current 25 responses of at least four oocytes per experiment are represented in the Figures. All curve fits were done using Sigma Plot software (Jandel Scientific) with the following equations:

(i) Response (for concentration-response curves)
= [(max-min)/(1+ (EC₅₀/concentration)ⁿ)]+min, and

30 (ii) Response (for concentration-inhibition curves) = [(max-min)/(1+ (concentration/IC₅₀)ⁿ)]+min.

Atropine sulfate, (-)-nicotine ditartrate, (+)-muscarine chloride, strychnine hydrochloride and oxotremorine-M w r obtained from RBI (Natick, MA),

kappa-Bungarotoxin was donated by Dr. V. Chiappinelli (St. Louis University Medical Center, St. Louis, MO). All other drugs were obtained from Sigma Chemical Co. (St. Louis, MO). Drugs were dissolved in frog Ringer's solution.

5 Bovine serum albumin (100 mg/ml) was added to the toxin solutions.

EXAMPLE V

In situ Hybridization

Experiments were carried out using mid-sagittal
10 sections of rat E16 embryos (Hybrid-ready tissue, Novagen, Madison, WI) and 20 μ m thick coronal sections of adult rat brains, according to the protocol described by Simmons, et al. in *J. Histotechnol.*, 12, 169-181, 1989. Either 35 S- or 32 P-labeled RNA probes were derived from the alpha9 cDNA
15 (e.g., nucleotides 283 to 806, Figure 1; i.e., nucleotides 455 to 979 of SEQ ID NO:1). Hybridization was carried out at 65°C and final washes were carried out at 72°C in 0.1 x SSC (1 x SSC is 180mM NaCl and 17mM sodium citrate, pH 7.0). Slides were dipped in Kodak NTB-2 emulsion,
20 developed in Kodak D19 after 3 weeks exposure at 4°C and subsequently Nissl stained.

EXAMPLE VI

Amplification Reactions

Tissues were obtained from adult Sprague Dawley
25 rats. The animals were decapitated and the tissues were quickly dissected and immersed in liquid nitrogen. Total RNA was isolated according to Chomczynski and Sacchi (see *Analytical Biochem.*, 162, 156-159, 1987), using the TRIzol reagent (Gibco BRL, Gaithersburg, MD). First strand cDNA
30 was synthesized from 2 μ g of total RNA with the Superscript Preamplification System (Gibco BRL). An aliquot containing 50ng of cDNA was used as template in amplification reactions. The following specific primers for alpha9 were

employed: sense primer, nucleotides 778 to 802; antisense primer, nucleotide 1353 to 1327 (Figure 1; nucleotides 951 to 975 and nucleotides 1526 to 1500, respectively, of SEQ ID NO:1). The predicted fragment spans one intron-exon boundary. A 573 base pair band is expected in the case of amplification from cDNA, whereas a fragment of ~1450 bp would result from the amplification of contaminant genomic DNA. Reactions were done in the following reaction mixture: 5U of Taq DNA polymerase, 5U of Taq enhancer (Stratagene, La Jolla, CA), 5 μ M of each primer, 50 μ M each of dATP, dGTP, dCTP and dTTP, 20mM Tris-HCl, pH 8.5, 10mM $(\text{H}_4\text{N})_2\text{SO}_4$, 2mM MgSO₄, 0.1% Triton X-100 and 0.1 mg/ml bovine serum albumin. Cycle parameters were: 2 min. at 95°C followed by 34 cycles each of 1 min. at 55°C, 1 min. at 72°C, 30 sec. at 95°C and a final cycle of 1 min. at 55°C, 5 min. at 72°C.

EXAMPLE VII

Detection of alpha9 Transcripts in Rat Cochlea

To determine if the alpha9 gene is expressed in rat cochlea, amplification reactions were performed on cDNA reverse transcribed from cochlear total RNA. As described in Example V, two primers specific for the alpha9 sequence were employed in order to amplify a fragment that spans an intron-exon boundary and additionally to avoid possible amplification from genomic DNA. Since alpha9 is present in the rat olfactory epithelium, cDNA obtained from this tissue was used as a positive control. Sciatic nerve cDNA was included to rule out the possibility that, with the parameters used for the amplification reactions, very small amounts of transcripts would be detected in any tissue studied. Whereas no DNA was amplified from the sciatic nerve using specific primers for alpha9 (Figure 8), both alpha3 and alpha4 subunits could be detected in this tissue with the respective specific primers.

A fragment of the expected size (573 bp) for amplification from alpha9 cDNA was obtained in the rat cochlea. Restriction endonuclease analysis of the fragment with *Acc*I, *Hinf*I and *Nco*I, further confirmed that this 5 fragment had been derived from alpha9 transcripts.

Although the invention has been described with reference to the specific embodiments, those skilled in the art will readily appreciate that the specific experiments taught hereinabove are only illustrative of the invention. 10 It should be understood that various modifications and variations can be made without departing from the spirit and scope of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: ELGOYHEN, ANA BELEN
JOHNSON, DAVID S.
BOULTER, JAMES R.
HEINEMANN, STEPHEN P.

(ii) TITLE OF INVENTION: CLONING AND EXPRESSION OF A NOVEL
ACETYLCHOLINE-GATED ION CHANNEL RECEPTOR

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

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(F) ZIP: 90071

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/278,635
(B) FILING DATE: 21-JUL-1994
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: REITER, STEPHEN E.
(B) REGISTRATION NUMBER: 31,192
(C) REFERENCE/DOCKET NUMBER: P41 9771

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 619-546-4737
(B) TELEFAX: 619-546-9392

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1938 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: ALPHA 9

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 89..1525

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

| | |
|--|-----|
| CGGTGGCAGT GAGGGTGTGTT TGAGCCCTTC ACAGACAGAA GTGGGAGTCC TCGCTGTCTG | 60 |
| CCTGACACAT TCTACATGTT GGGAAAAG ATG AAC CGG CCC CAT TCC TGC CTC | 112 |
| Met Asn Arg Pro His Ser Cys Leu | |
| 1 5 | |
| TCC TTT TGC TGG ATG TAT TTT GCT GCT TCT GGA ATC AGA GCC GTA GAG | 160 |
| Ser Phe Cys Trp Met Tyr Phe Ala Ala Ser Gly Ile Arg Ala Val Glu | |
| 10 15 20 | |
| ACA GCA AAT GGG AAA TAT GCT CAG AAA TTG TTC AGC GAT CTT TTT GAA | 208 |
| Thr Ala Asn Gly Lys Tyr Ala Gln Lys Leu Phe Ser Asp Leu Phe Glu | |
| 25 30 35 40 | |
| GAC TAC TCC AGT GCT CTG CGT CCA GTC GAG GAT ACG GAC GCG GTG CTG | 256 |
| Asp Tyr Ser Ser Ala Leu Arg Pro Val Glu Asp Thr Asp Ala Val Leu | |
| 45 50 55 | |
| AAT GTT ACA CTG CAG GTC ACG CTC TCC CAG ATA AAG GAC ATG GAC GAG | 304 |
| Asn Val Thr Leu Gln Val Thr Leu Ser Gln Ile Lys Asp Met Asp Glu | |
| 60 65 70 | |
| AGA AAC CAG ATT CTG ACA GCC TAT CTA TGG ATC CGC CAA ACC TGG CAC | 352 |
| Arg Asn Gln Ile Leu Thr Ala Tyr Leu Trp Ile Arg Gln Thr Trp His | |
| 75 80 85 | |
| GAT GCG TAC CTC ACG TGG GAT CGA GAC CAG TAT GAT AGG CTG GAC TCC | 400 |
| Asp Ala Tyr Leu Thr Trp Asp Arg Asp Gln Tyr Asp Arg Leu Asp Ser | |
| 90 95 100 | |
| ATC AGG ATT CCC AGC GAT CTG GTG TGG AGG CCG GAC ATT GTC CTA TAC | 448 |
| Ile Arg Ile Pro Ser Asp Leu Val Trp Arg Pro Asp Ile Val Leu Tyr | |
| 105 110 115 120 | |
| AAC AAG GCT GAC GAT GAG TCT TCA GAG CCT GTG AAC ACC AAT GTG GTG | 496 |
| Asn Lys Ala Asp Asp Glu Ser Ser Glu Pro Val Asn Thr Asn Val Val | |
| 125 130 135 | |
| CTG CGA TAT GAT GGG CTC ATC ACC TGG GAC TCA CCG GCC ATC ACC AAA | 544 |
| Leu Arg Tyr Asp Gly Leu Ile Thr Trp Asp Ser Pro Ala Ile Thr Lys | |
| 140 145 150 | |
| AGC TCC TGT GTG GTG GAT GTC ACC TAC TTC CCT TTT GAC AGC CAG CAG | 592 |
| Ser Ser Cys Val Val Asp Val Thr Tyr Phe Pro Phe Asp Ser Gln Gln | |
| 155 160 165 | |
| TGC AAC CTG ACC TTT GGC TCC TGG ACC TAC AAT GGA AAC CAG GTG GAC | 640 |
| Cys Asn Leu Thr Phe Gly Ser Trp Thr Tyr Asn Gly Asn Gln Val Asp | |
| 170 175 180 | |
| ATA TTC AAT GCC CTG GAC AGC GGT GAC CTC TCT GAC TTC ATT GAA GAT | 688 |
| Ile Phe Asn Ala Leu Asp Ser Gly Asp Leu Ser Asp Phe Ile Glu Asp | |
| 185 190 195 200 | |
| GTG GAA TGG GAG GTC CAT GGC ATG CCT GCT GTA AAG AAC GTC ATC TCC | 736 |
| Val Glu Trp Glu Val His Gly Met Pro Ala Val Lys Asn Val Ile Ser | |
| 205 210 215 | |
| TAT GGC TGC TGC TCC GAG CCT TAC CCA GAT GTC ACC TTC ACT CTC CTT | 784 |
| Tyr Gly Cys Cys Ser Glu Pro Tyr Pro Asp Val Thr Phe Thr Leu Leu | |
| 220 225 230 | |
| CTG AAG AGG AGG TCC TCC TTC TAC ATC GTC AAC CTC CTC ATC CCT TGC | 832 |
| Leu Lys Arg Arg Ser Ser Phe Tyr Ile Val Asn Leu Leu Ile Pro Cys | |
| 235 240 245 | |

| | |
|--|---------------------|
| GTC CTC ATA TCG TTC CTC GCT CCG TTG AGT TTC TAT CTC CCA GCA GCC Val Leu Ile Ser Phe L u Ala Pro Leu Ser Phe Tyr Leu Pro Ala Ala 250 255 260 | 880 |
| TCT GGG GAG AAG GTC TCT CTG GGA GTG ACC ATC CTA TTG GCC ATG ACT Ser Gly Glu Lys Val Ser Leu Gly Val Thr Ile Leu Leu Ala Met Thr 265 270 275 280 | 928 |
| G TG TTT CAG CTA ATG GTG GCA GAG ATC ATG CCA GCC TCA GAA AAT GTC Val Phe Gln Leu Met Val Ala Glu Ile Met Pro Ala Ser Glu Asn Val 285 290 295 | 976 |
| CCT CTG ATA GGA AAA TAC TAC ATA GCT ACC ATG GCC TTG ATC ACT GCC Pro Leu Ile Gly Lys Tyr Tyr Ile Ala Thr Met Ala Leu Ile Thr Ala 300 305 310 | 1024 |
| TCC ACA GCC CTT ACC ATC ATG GTG ATG AAT ATT CAC TTC TGT GGA GCT Ser Thr Ala Leu Thr Ile Met Val Met Asn Ile His Phe Cys Gly Ala 315 320 325 | 1072 |
| GAG GCA CGG CCA GTG CCA CAC TGG GCC AAG GTG GTC ATC CTG AAG TAC Glu Ala Arg Pro Val Pro His Trp Ala Lys Val Val Ile Leu Lys Tyr 330 335 340 | 1120 |
| ATG TCC AGG ATC TTG TTT GTC TAC GAT GTG GGT GAG AGC TGC CTT AGT Met Ser Arg Ile Leu Phe Val Tyr Asp Val Gly Glu Ser Cys Leu Ser 345 350 355 360 | 1168 |
| CCC CGC CAC AGC CAG GAG CCA GAA CAA GTC ACG AAG GTT TAT AGC AAA Pro Arg His Ser Gln Glu Pro Glu Gln Val Thr Lys Val Tyr Ser Lys 365 370 375 | 1216 |
| CTC CCA GAA TCC AAC CTG AAA ACG TCC AGA AAC AAA GAC CTT TCC AGA Leu Pro Glu Ser Asn Leu Lys Thr Ser Arg Asn Lys Asp Leu Ser Arg 380 385 390 | 1264 |
| AAG AAG GAA GTA ACA AAA CTC TTA AAG AAT GAC CTG GGG TAC CAG GGT Lys Lys Glu Val Arg Lys Leu Leu Lys Asn Asp Leu Gly Tyr Gln Gly 395 400 405 | 1312 |
| GGG ATC CCC CAG AAT ACT GAC AGT TAT TGT GCA CGC TAT GAA GCA CTG Gly Ile Pro Gln Asn Thr Asp Ser Tyr Cys Ala Arg Tyr Glu Ala Leu 410 415 420 | 1360 |
| GCG AAA AAT ATC GAA TAC ATT GCC AAG TGC CTC AAG GAC CAC AAG GCC Ala Lys Asn Ile Glu Tyr Ile Ala Lys Cys Leu Lys Asp His Lys Ala 425 430 435 440 | 1408 |
| ACC AAC TCC AAG GGC AGC GAG TGG AAG AAG GTC GCC AAA GTC ATA GAC Thr Asn Ser Lys Gly Ser Glu Trp Lys Lys Val Ala Lys Val Ile Asp 445 450 455 | 1456 |
| CGT TTC TTC ATG TGG ATT TTC TTT GCT ATG GTG TTT GTC ATG ACC GTC Arg Phe Phe Met Trp Ile Phe Ala Met Val Phe Val Met Thr Val 460 465 470 | 1504 |
| TTG ATC ATA GCA AGA GCA GAT TAGCAGGAAA GAGGAGTGGG CTGGTAGGCA Leu Ile Ile Ala Arg Ala Asp 475 | 1555 |
| TTTAGAGATT TGGGGAAAAC CCAATAAAAT CACCTGAGAT CTGCCCCAGC GTGTGAGTTC AGCTGCTGTT CATACTATAAT TTAGGGATA GGTTGCGTAT GCTTTTATTCTA CTAACCTCAA TCAATATCCT AGTTACATGT CAGGTTAAAT CAAGCAGGAG ATGCAAGGTT TCAAGGGTAA AGGGCTGGAG GAAGAGAGTT AGAAAGGACC CTTTCACAGG CTCCCATGAA GGGGAGTGGT | 1615 1675 1735 1795 |

| | |
|--|------|
| GGCCTTCAGT TTATGTAATT ATCTCTTAT TATTGTAGAC AACAAAGCAC AGTGTATTCC | 1855 |
| TGCTTAAGAT TTAAAGCAAG AAAAGACAAA ACAAAATTCAT TCTCTTAGTC CTTAATAAAA | 1915 |
| CTTTTTTTT TAAACAAAAA AAA | 1938 |

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 479 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

| | |
|---|--|
| Met Asn Arg Pro His Ser Cys Leu Ser Phe Cys Trp Met Tyr Phe Ala | |
| 1 5 10 15 | |
| Ala Ser Gly Ile Arg Ala Val Glu Thr Ala Asn Gly Lys Tyr Ala Gln | |
| 20 25 30 | |
| Lys Leu Phe Ser Asp Leu Phe Glu Asp Tyr Ser Ser Ala Leu Arg Pro | |
| 35 40 45 | |
| Val Glu Asp Thr Asp Ala Val Leu Asn Val Thr Leu Gln Val Thr Leu | |
| 50 55 60 | |
| Ser Gln Ile Lys Asp Met Asp Glu Arg Asn Gln Ile Leu Thr Ala Tyr | |
| 65 70 75 80 | |
| Leu Trp Ile Arg Gln Thr Trp His Asp Ala Tyr Leu Thr Trp Asp Arg | |
| 85 90 95 | |
| Asp Gln Tyr Asp Arg Leu Asp Ser Ile Arg Ile Pro Ser Asp Leu Val | |
| 100 105 110 | |
| Trp Arg Pro Asp Ile Val Leu Tyr Asn Lys Ala Asp Asp Glu Ser Ser | |
| 115 120 125 | |
| Glu Pro Val Asn Thr Asn Val Val Leu Arg Tyr Asp Gly Leu Ile Thr | |
| 130 135 140 | |
| Trp Asp Ser Pro Ala Ile Thr Lys Ser Ser Cys Val Val Asp Val Thr | |
| 145 150 155 160 | |
| Tyr Phe Pro Phe Asp Ser Gln Gln Cys Asn Leu Thr Phe Gly Ser Trp | |
| 165 170 175 | |
| Thr Tyr Asn Gly Asn Gln Val Asp Ile Phe Asn Ala Leu Asp Ser Gly | |
| 180 185 190 | |
| Asp Leu Ser Asp Phe Ile Glu Asp Val Glu Trp Glu Val His Gly Met | |
| 195 200 205 | |
| Pro Ala Val Lys Asn Val Ile Ser Tyr Gly Cys Cys Ser Glu Pro Tyr | |
| 210 215 220 | |
| Pro Asp Val Thr Phe Thr Leu Leu Leu Lys Arg Arg Ser Ser Phe Tyr | |
| 225 230 235 240 | |
| Ile Val Asn Leu Leu Ile Pro Cys Val Leu Ile Ser Phe Leu Ala Pro | |
| 245 250 255 | |

Leu Ser Ph Tyr Leu Pro Ala Ala Ser Gly Glu Lys Val Ser Leu Gly
260 265 270

Val Thr Ile Leu Leu Ala Met Thr Val Phe Gln Leu Met Val Ala Glu
275 280 285

Ile Met Pro Ala Ser Glu Asn Val Pro Leu Ile Gly Lys Tyr Tyr Ile
290 295 300

Ala Thr Met Ala Leu Ile Thr Ala Ser Thr Ala Leu Thr Ile Met Val
305 310 315 320

Met Asn Ile His Phe Cys Gly Ala Glu Ala Arg Pro Val Pro His Trp
325 330 335

Ala Lys Val Val Ile Leu Lys Tyr Met Ser Arg Ile Leu Phe Val Tyr
340 345 350

Asp Val Gly Glu Ser Cys Leu Ser Pro Arg His Ser Gln Glu Pro Glu
355 360 365

Gln Val Thr Lys Val Tyr Ser Lys Leu Pro Glu Ser Asn Leu Lys Thr
370 375 380

Ser Arg Asn Lys Asp Leu Ser Arg Lys Lys Glu Val Arg Lys Leu Leu
385 390 395 400

Lys Asn Asp Leu Gly Tyr Gln Gly Gly Ile Pro Gln Asn Thr Asp Ser
405 410 415

Tyr Cys Ala Arg Tyr Glu Ala Leu Ala Lys Asn Ile Glu Tyr Ile Ala
420 425 430

Lys Cys Leu Lys Asp His Lys Ala Thr Asn Ser Lys Gly Ser Glu Trp
435 440 445

Lys Lys Val Ala Lys Val Ile Asp Arg Phe Phe Met Trp Ile Phe Phe
450 455 460

Ala Met Val Phe Val Met Thr Val Leu Ile Ile Ala Arg Ala Asp
465 470 475

What is claimed is:

1. Isolated nucleic acid encoding an alpha9 nicotinic acetylcholine receptor (nAChR) subunit.
2. Isolated nucleic acid according to Claim 1, wherein said nucleic acid comprises DNA.
3. DNA according to Claim 2, wherein said DNA is a cDNA.
4. DNA according to Claim 2, wherein said DNA encodes the amino acid sequence set forth in SEQ ID NO:2.
5. DNA according to Claim 2, wherein said DNA hybridizes under high stringency conditions to the nucleotide sequence set forth in SEQ ID NO:1.
6. DNA according to Claim 2, wherein the nucleotide sequence of said DNA is substantially the same as the nucleotide sequence set forth in SEQ ID NO:1.
7. A vector comprising DNA according to Claim 2.
8. A host cell containing a vector according to Claim 7.
9. A host cell according to Claim 8, wherein said cell expresses functional nACh receptor comprising at least one alpha9 nAChR subunit.
10. A host cell according to Claim 9, wherein said receptor is homomeric.
11. A host cell according to Claim 9, wherein said receptor is heteromeric.

12. A nucleic acid probe comprising at least 14 contiguous nucleotides according to Claim 2.

13. Isolated mRNA complementary to DNA according to Claim 2.

14. An antisense oligonucleotide that specifically binds to and modulates translation of mRNA according to Claim 13.

15. Isolated alpha₉ nicotinic acetylcholine receptor subunit, and biologically active fragments thereof.

16. Isolated alpha₉ nicotinic acetylcholine receptor subunit according to Claim 15 wherein said subunit has the same amino acid sequence set forth in SEQ ID NO:2.

17. Isolated alpha₉ nicotinic acetylcholine receptor subunit according to Claim 15 wherein said subunit has substantially the same amino acid sequence as that set forth in SEQ ID NO:2.

18. A functional nicotinic acetylcholine receptor expressed recombinantly in a host cell, said receptor characterized by:

(i) being expressed in the pituitary, olfactory epithelium, cochlea and tongue;

(ii) being activated by acetylcholine but blocked by nicotine and muscarine, and

(iii) comprising at least one alpha₉ acetylcholine receptor subunit.

19. A receptor according to Claim 18 wherein said alpha₉ nicotinic acetylcholine receptor subunit has the same amino acid sequence set forth in SEQ ID NO:2.

20. A receptor according to Claim 18 wherein said alpha₉ nicotinic acetylcholine receptor subunit has substantially the same amino acid sequence as that set forth in SEQ ID NO:2.

21. An antibody that specifically binds to an alpha₉ nicotinic acetylcholine receptor subunit.

22. An antibody according to Claim 21, wherein said antibody is a monoclonal antibody.

23. An antibody according to Claim 21, wherein said antibody is a polyclonal antibody.

24. A composition comprising:

an amount of an oligonucleotide according to Claim 14 effective to modulate expression of a nicotinic acetylcholine receptor comprising at least one alpha₉ subunit, and

an acceptable hydrophobic carrier capable of passing through a cell membrane.

25. A composition comprising an amount of the antibody according to Claim 21 effective to block binding of naturally occurring ligands to a nicotinic acetylcholine receptor comprising at least one alpha₉ subunit and an acceptable carrier.

26. A transgenic non-human mammal expressing DNA encoding an alpha₉ nicotinic acetylcholine receptor subunit.

27. A transgenic non-human mammal according to Claim 26, wherein DNA encoding said receptor has been mutated such that the expressed receptor does not contain wild-type alpha9 nicotinic acetylcholine receptor subunits.

28. A transgenic non-human mammal according to Claim 26, wherein the transgenic non-human mammal is a mouse.

29. A method for identifying nucleic acids encoding alpha9 nicotinic acetylcholine receptor subunit(s), said method comprising:

contacting a sample containing a population of nucleic acids with a probe according to Claim 12, wherein said contacting is effected under high stringency hybridization conditions, and

identifying nucleic acids which hybridize to said probe.

30. A method for identifying compounds which bind to alpha9 nicotinic acetylcholine receptor subunit(s), said method comprising contacting cells according to Claim 9 with test compound and identifying compounds which bind thereto.

31. A bioassay for identifying compounds which modulate the activity of nicotinic acetylcholine receptors comprising at least one alpha9 subunit, said method comprising:

contacting cells according to Claim 9 with at least one compound wherein the ability of said compound to modulate the ion channel of the receptor is unknown; and thereafter

monitoring the cells for changes in ion channel activity.

32. A method according to Claim 31 wherein said cells are contacted with at least one potential agonist.

33. A method according to Claim 31 wherein said cells are contacted with a constant amount of agonist and increasing amounts of at least one potential antagonist.

34. A bioassay for identifying compounds which modulate the regulatory regions(s) of the alpha9 nicotinic acetylcholine receptor subunit gene, said method comprising:

transforming mammalian cells with a nucleic acid construct comprising at least a portion of the regulatory region of the alpha9 gene, wherein said portion of the regulatory gene is operatively associated with a reporter gene;

contacting said transformed cells with at least one compound wherein the ability of the compound to modulate the regulatory region of the alpha9 receptor subunit gene is unknown; and

monitoring said cells for expression of said reporter gene.

1 / 14

-153

-120 ACA GAA GTG GGA GTC CTC GCT GTC TGC CTG ACA CAT TCT
 -22

1 GTA GAG ACA GCA AAT GGG AAA TAT GCT CAG AAA TTG TTC
 1 Val Glu Thr Ala Asn Gly Lys Tyr Ala Gln Lys Leu Phe

121 ACG CTC TCC CAG ATA AAG GAC ATG GAC GAG AGA AAC CAG
 41 Thr Leu Ser Gln Ile Lys Asp Met Asp Glu Arg Asn Gln

241 GAC TCC ATC AGG ATT CCC AGC GAT CTG GTG TGG AGG CCG
 81 Asp Ser Ile Arg Ile Pro Ser Asp Leu Val Trp Arg Pro

361 ATC ACC TGG GAC TCA CCG GCC ATC ACC AAA AGC TCC TGT
 121 Ile Thr Trp Asp Ser Pro Ala Ile Thr Lys Ser Ser Cys

481 GTG GAC ATA TTC AAT GCC CTG GAC AGC GGT GAC CTC TCT
 161 Val Asp Ile Phe Asn Ala Leu Asp Ser Gly Asp Leu Ser

601 CCT TAC CCA GAT GTC ACC TTC ACT CTC CTT CTG AAG AGG
 201 Pro Tyr Pro Asp Val Thr Phe Thr Leu Leu Lys Arg

721 GCA GCC TCT CGG GAG AAG GTC TCT CTG GGA GTG ACC ATC
 241 Ala Ala Ser Gly Glu Lys Val Ser Leu Gly Val Thr Ile

841 TAC ATA GCT ACC ATG GCC TTG ATC ACT GCC TCC ACA GCC
 281 Tyr Ile Ala Thr Met Ala Leu Ile Thr Ala Ser Thr Ala
 -----MSR III-----

961 AAG TAC ATG TCC AGG ATC TTG TTT GTC TAC GAT GTG GGT
 321 Lys Tyr Met Ser Arg Ile Leu Phe Val Tyr Asp Val Gly

1081 AAA ACG TCC AGA AAC AAA GAC CTT TCC AGA AAG AAG GAA
 361 Lys Thr Ser Arg Asn Lys Asp Leu Ser Arg Lys Lys Glu

1201 GCA CTG GCG AAA AAT ATC GAA TAC ATT GCC AAG TGC CTC
 401 Ala Leu Ala Lys Asn Ile Glu Tyr Ile Ala Lys Cys Leu

1321 TTC TTT GCT ATG GTG TTT GTC ATG ACC GTC TTG ATC ATA
 441 Phe Phe Ala Met Val Phe Val Met Thr Val Leu Ile Ile
 ---MSR IV-----

1441 TCT GCC CCA GCG TGT GAG TTC AGC TGC TGT TCA TAC ATA
 1561 GAT GCA AGG TTT CAA GGG TAA AGG GCT GGA GGA AGA GAG
 1681 CAA CAA AGC ACA GTG TAT TCC TGC TTA AGA TTT AAA GCA

FIG. 1A

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ACA TGT TGG GAA AAG ATG AAC CGG CCC CAT TCC TGC CTC TCC
 Met Asn Arg Pro His Ser Cys Leu Ser

 AGC GAT CTT TTT GAA GAC TAC TCC AGT GCT CTG CGT CCA GTC
 Ser Asp Leu Phe Glu Asp Tyr Ser Ser Ala Leu Arg Pro Val

ATT CTG ACA GCC TAT CTA TGG ATC CGC CAA ACC TGG CAC GAT
 Ile Leu Thr Ala Tyr Leu Trp Ile Arg Gln Thr Trp His Asp

↓
 GAC ATT GTC CTA TAC AAC AAG GCT GAC GAT GAG TCT TCA GAG
 Asp Ile Val Leu Tyr Asn Lys Ala Asp Asp Glu Ser Ser Glu

GTG GTG GAT GTC ACC TAC TTC CCT TTT GAC AGC CAG CAG TGC
 Val Val Asp Val Thr Tyr Phe Pro Phe Asp Ser Gln Gln Cys

GAC TTC ATT GAA GAT GTG GAA TGG GAG GTC CAT GGC ATG CCT
 Asp Phe Ile Glu Asp Val Glu Trp Glu Val His Gly Met Pro

AGG TCC TCC TTC TAC ATC GTC AAC CTC CTC ATC CCT TGC GTC
 Arg Ser Ser Phe Tyr Ile Val Asn Leu Leu Ile Pro Cys Val

 CTA TTG GCC ATG ACT GTG TTT CAG CTA ATG GTG GCA GAG ATC
 Leu Leu Ala Met Thr Val Phe Gln Leu Met Val Ala Glu Ile

-----MSR II-----

 CTT ACC ATC ATG GTG ATG AAT ATT CAC TTC TGT GGA GCT GAG
 Leu Thr Ile Met Val Met Asn Ile His Phe Cys Gly Ala Glu

 GAG AGC TGC CTT AGT CCC CGC CAC AGC CAG GAG CCA GAA CAA
 Glu Ser Cys Leu Ser Pro Arg His Ser Gln Glu Pro Glu Gln

 GTA AGA AAA CTC TTA AAG AAT GAC CTG GGG TAC CAG GGT GGG
 Val Arg Lys Leu Leu Lys Asn Asp Leu Gly Tyr Gln Gly Gly

 AAG GAC CAC AAG GCC ACC AAC TCC AAG GGC AGC GAG TGG AAG
 Lys Asp His Lys Ala Thr Asn Ser Lys Gly Ser Glu Trp Lys

 GCA AGA GCA GAT TAG CAG GAA AGA GGA GTG GGC TGG TAG GCA
 Ala Arg Ala Asp

 ATT TAG GGG ATA GGT TGC GTA TGC TTT TAT TCC TAA CTT CAA
 TTA GAA AGG ACC CTT TCA CAG GCT CCC ATG AAG GGG AGT GGT
 AGA AAA GAC AAA ACA AAT TCA TTC TCT TAG TCC TTA ATA AAA

FIG. 1B

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| | |
|---|------|
| GGT GGC AGT GAG GGT GTT TTG AGC CCT TCA CAG | -121 |
| ↓ | |
| TTT TGC TGG ATG TAT TTT GCT GCT TCT GGA ATC AGA GCC | -1 |
| Phe Cys Trp Met Tyr Phe Ala Ala Ser Gly Ile Arg Ala | -1 |
| ----- -SIGNAL PEPTIDE----- | |
| GAG GAT ACG GAC GCG GTC CTG AAT GTT ACA CTG CAG GTC | 120 |
| Glu Asp Thr Asp Ala Val Leu Asn Val Thr Leu Gln Val | 40 |
| GCG TAC CTC ACG TGG GAT CGA GAC CAG TAT GAT AGG CTG | 240 |
| Ala Tyr Leu Thr Trp Asp Arg Asp Gln Tyr Asp Arg Leu | 80 |
| CCT GTG AAC ACC AAT GTG GTG CTG CGA TAT GAT GGG CTC | 360 |
| Pro Val Asn Thr Asn Val Val Leu Arg Tyr Asp Gly Leu | 120 |
| AAC CTG ACC TTT GGC TCC TGG ACC TAC AAT GGA AAC CAG | 480 |
| Asn Leu Thr Phe Gly Ser Trp Thr Tyr Asn Gly Asn Gln | 160 |
| GCT GTA AAG AAC GTC ATC TCC TAT GGC TGC TGC TCC GAG | 600 |
| Ala Val Lys Asn Val Ile Ser Tyr Gly Cys Cys Ser Glu | 200 |
| CTC ATA TCG TTC CTC GCT CCG TTG AGT TTC TAT CTC CCA | 720 |
| Leu Ile Ser Phe Leu Ala Pro Leu Ser Phe Tyr Leu Pro | 240 |
| -----MSR I----- | |
| ↓ | |
| ATG CCA GCC TCA GAA AAT GTC CCT CTG ATA GGA AAA TAC | 840 |
| Met Pro Ala Ser Glu Asn Val Pro Leu Ile Gly Lys Tyr | 280 |
| ----- | |
| GCA CGG CCA GTG CCA CAC TGG GCC AAG GTG GTC ATC CTG | 960 |
| Ala Arg Pro Val Pro His Trp Ala Lys Val Val Ile Leu | 320 |
| ----- | |
| GTC ACG AAG GTT TAT AGC AAA CTC CCA GAA TCC AAC CTG | 1080 |
| Val Thr Lys Val Tyr Ser Lys Leu Pro Glu Ser Asn Leu | 360 |
| ATC CCC CAG AAT ACT GAC AGT TAT TGT GCA CGC TAT GAA | 1200 |
| Ile Pro Gln Asn Thr Asp Ser Tyr Cys Ala Arg Tyr Glu | 400 |
| AAG GTC GCC AAA GTC ATA GAC CGT TTC TTC ATG TGG ATT | 1320 |
| Lys Val Ala Lys Val Ile Asp Arg Phe Phe Met Trp Ile | 440 |
| ----- | |
| TTT AGA GAT TTG GGG AAA ACC CAA TAA AAT CAC CTG AGA | 1440 |
| | 457 |
| ----- | |
| TCA ATA TCC TAG TTA CAT GTC AGG TTA AAT CAA GCA GGA | 1560 |
| GGC CTT CAG TTT ATG TAA TTA TCT CTT TAT TGT AGA | 1680 |
| CTT TTT TTT TTA AAC AAA AAA AA | 1784 |

FIG. 1C

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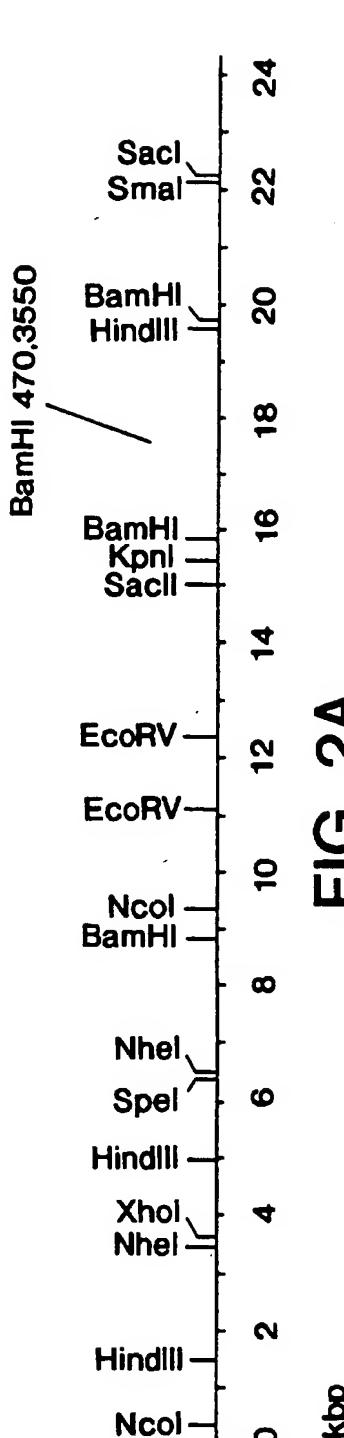


FIG. 2A

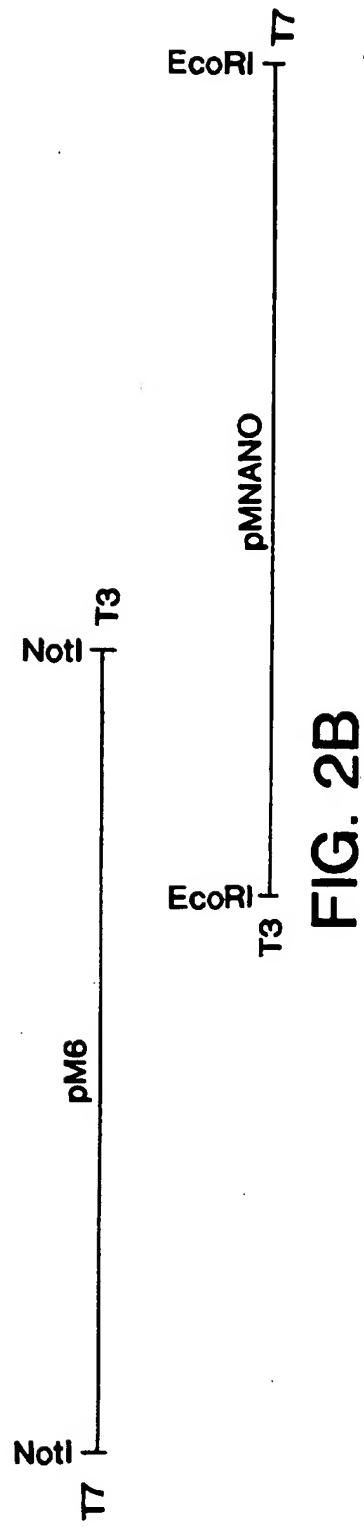


FIG. 2B

FIG. 3A

| ALPHA 1 | STSEAVPLIGKYLIFTMIVFVLASIIINIVMANTHRSPTSTHOMPFTMPEKTFIDITIPNTMPSRSDIKOEKRIFTEDIDISICKPQPMMGPHH |
|---------|--|
| ALPHA 2 | STISLNTPLIGEYLIFTMIVFVLASIIINIVMANTHRSPTSTHOMPFTMPEKTFIDITIPNTMPSRSDIKOEKRIFTEDIDISICKPQPMMGPHH |
| ALPHA 3 | STSIAVPLIGEYLIFTMIVFVLASIIINIVMANTHRSPTSTHOMPFTMPEKTFIDITIPNTMPSRSDIKOEKRIFTEDIDISICKPQPMMGPHH |
| ALPHA 4 | STSISLNTPLIGEYLIFTMIVFVLASIIINIVMANTHRSPTSTHOMPFTMPEKTFIDITIPNTMPSRSDIKOEKRIFTEDIDISICKPQPMMGPHH |
| ALPHA 5 | ATSSVPLIAGYFASTMIVFVLASIIINIVMANTHRSPTSTHOMPFTMPEKTFIDITIPNTMPSRSDIKOEKRIFTEDIDISICKPQPMMGPHH |
| ALPHA 6 | ATSSVPLIAGYFASTMIVFVLASIIINIVMANTHRSPTSTHOMPFTMPEKTFIDITIPNTMPSRSDIKOEKRIFTEDIDISICKPQPMMGPHH |
| ALPHA 7 | ATSSVPLIAGYFASTMIVFVLASIIINIVMANTHRSPTSTHOMPFTMPEKTFIDITIPNTMPSRSDIKOEKRIFTEDIDISICKPQPMMGPHH |
| ALPHA 8 | ATSSVPLIAGYFASTMIVFVLASIIINIVMANTHRSPTSTHOMPFTMPEKTFIDITIPNTMPSRSDIKOEKRIFTEDIDISICKPQPMMGPHH |
| ALPHA 9 | ATSSVPLIAGYFASTMIVFVLASIIINIVMANTHRSPTSTHOMPFTMPEKTFIDITIPNTMPSRSDIKOEKRIFTEDIDISICKPQPMMGPHH |

ALPHA 1 LDDICNOGLSPAPTPCNPTDCPTTSEVERKSPCPSPGSCPPKSSGAPMLIKARSLSVQHVSSEALEDGIRCRSRSIQYC
ALPHA 2 SP LIKPEVKSAIEGVKIAETMKSDOESNNAEEMW
ALPHA 3 EEEEDENICVAGLPDSMGMVLYCHGGHLIRAMEPETTPSOASEILLSPQIKALEGVVWIAEILRSEDADSUVREDW
ALPHA 4 EGYP CQDGTCGCHARRVKLSENFSANLRTSSSSSENAVLSLALSPEIIEKAIGSVKIAEMLKAONVAKIEQDDW
ALPHA 5 VSDQGASLANSKPTSSPTSLKARPSPQLPVSDOASPCKTCKEPSPVSPVTVLKGACTKAPPQHPLPLSPALTRAVEGVQWIAEILKAEDTDFSVREDW
ALPHA 6 GNLIXIGFRGLEGHICAPTPDSCGVVCGRLACSPTHDEILMGAHPSDGDPDLAKILEEVRYIANERFCODESEVTCSPW
ALPHA 7 SNGNDLTSYHNMENPCCPQNDLGSKSGKTCPLSEINNEHVKALMDTIPVTKILEEVQFIAERFKQDECEEICSEW
ALPHA 8 SP KKEPBEUINDIGYCCGCTBONTTSCAVERAIAVTEVWNGCCTTPTTATVWVQDQVWVQDQVWVQDQVWVQDQVW
ALPHA 9

| | |
|--------|--|
| ALPHA1 | YVAVYDRIILGVEMLVCLIGLTLAVPAGRLIELHOOG |
| ALPHA2 | YVAVYDRIIFLNLFLIVCFLIGLIGLPPFLAGMT |
| ALPHA3 | YVAVYDRIIPUWELVCLIGLIGLTCAGLFLOPLMARDT |
| ALPHA4 | YVAVYDRIIFLMLTIVCLIGLIGLPPFLAAC |
| ALPHA7 | PAACYDRLCIMAFTSFTLICLIGLIMSAPNFVEAVSKOPA |
| ALPHA8 | FMAAVDRLCLVFTLFLAICLFTLMSAPNPIEAVSKDFT |
| ALPHA9 | KWAKVLDRIFFWVIEFAMVFLVWVILARAD |

MSR IV —

FIG. 3B

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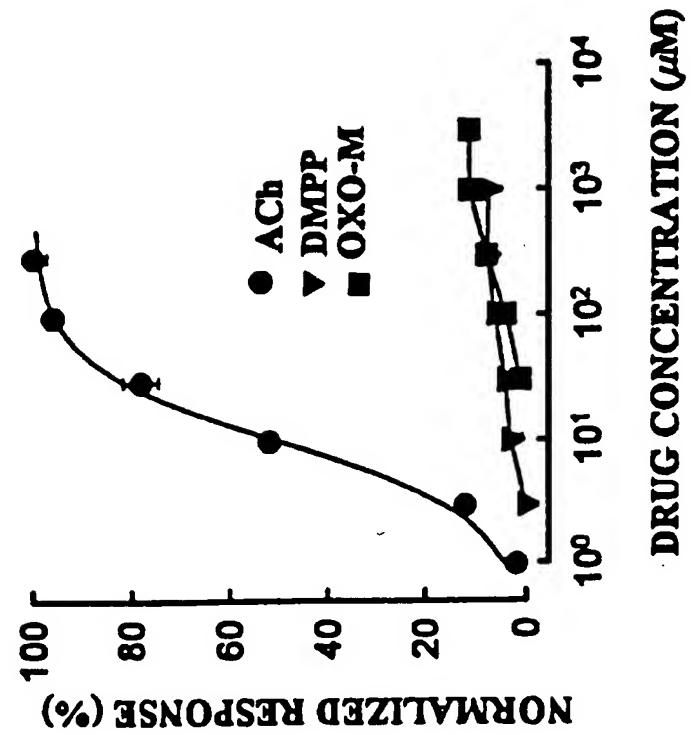


FIG. 4B

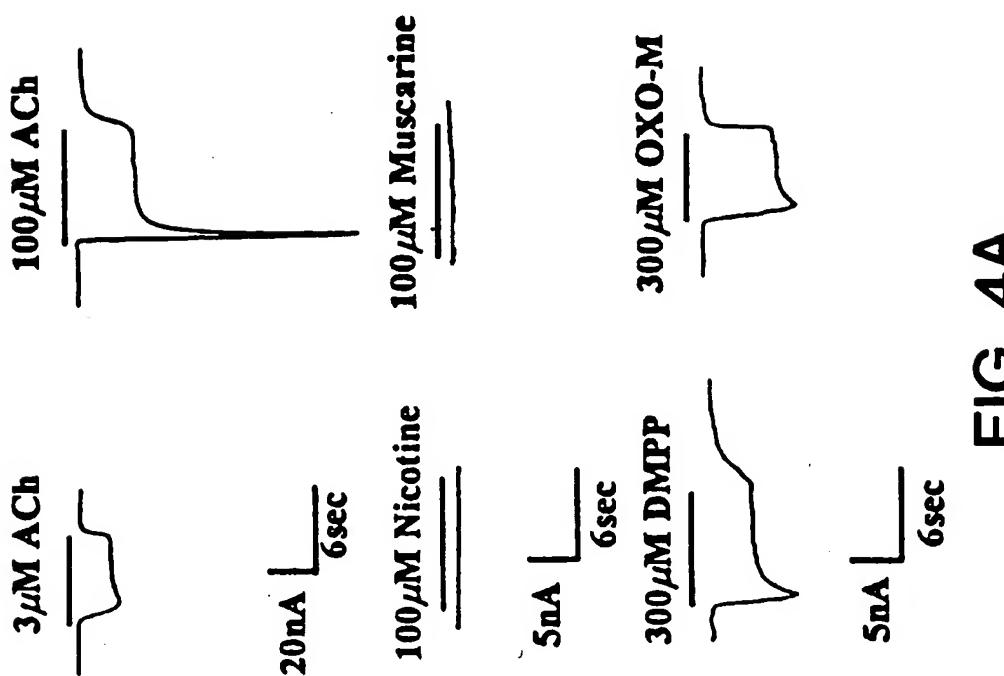


FIG. 4A

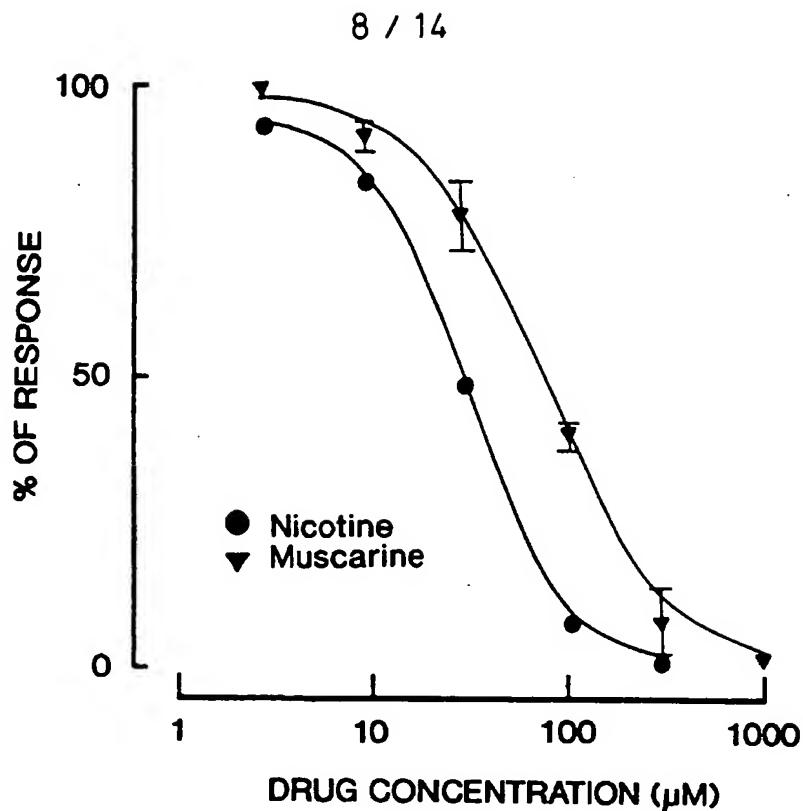


FIG. 5A

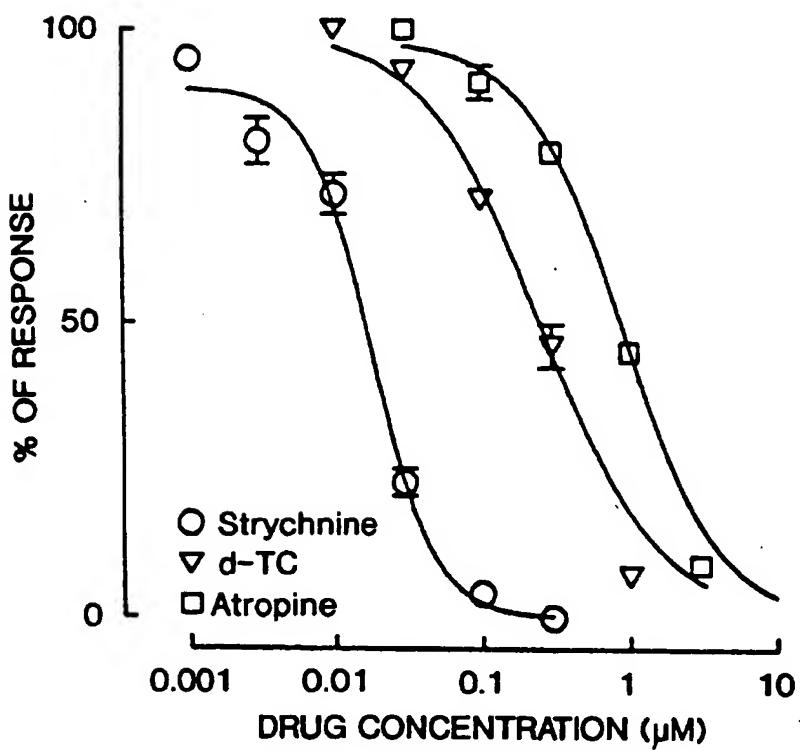


FIG. 5B

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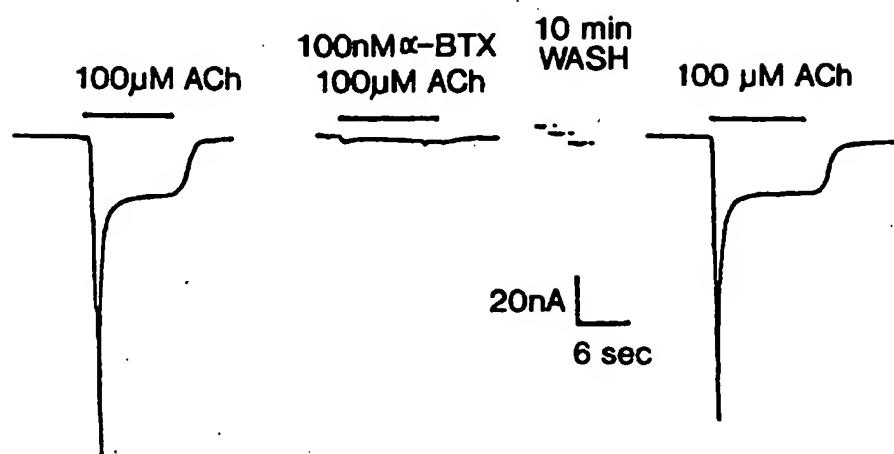


FIG. 6A

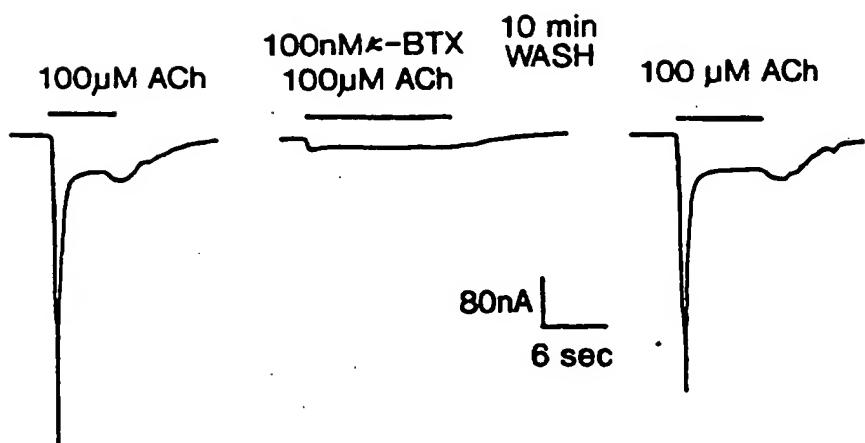


FIG. 6B

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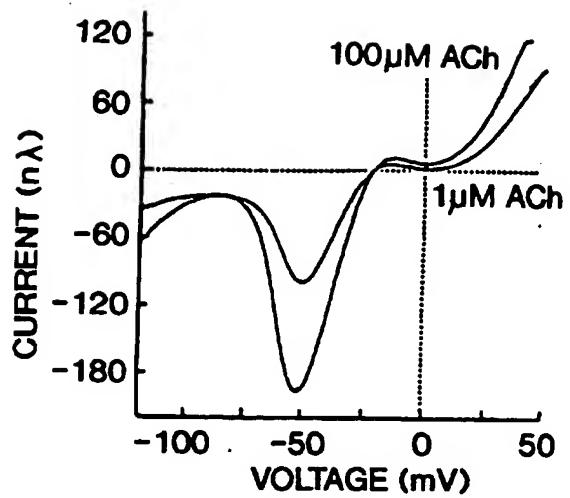


FIG. 7A

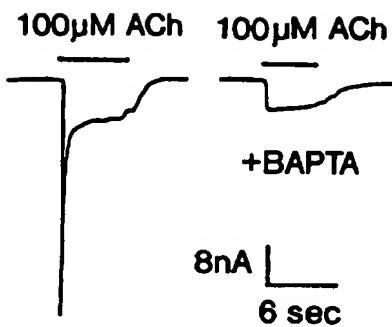


FIG. 7B



FIG. 7C

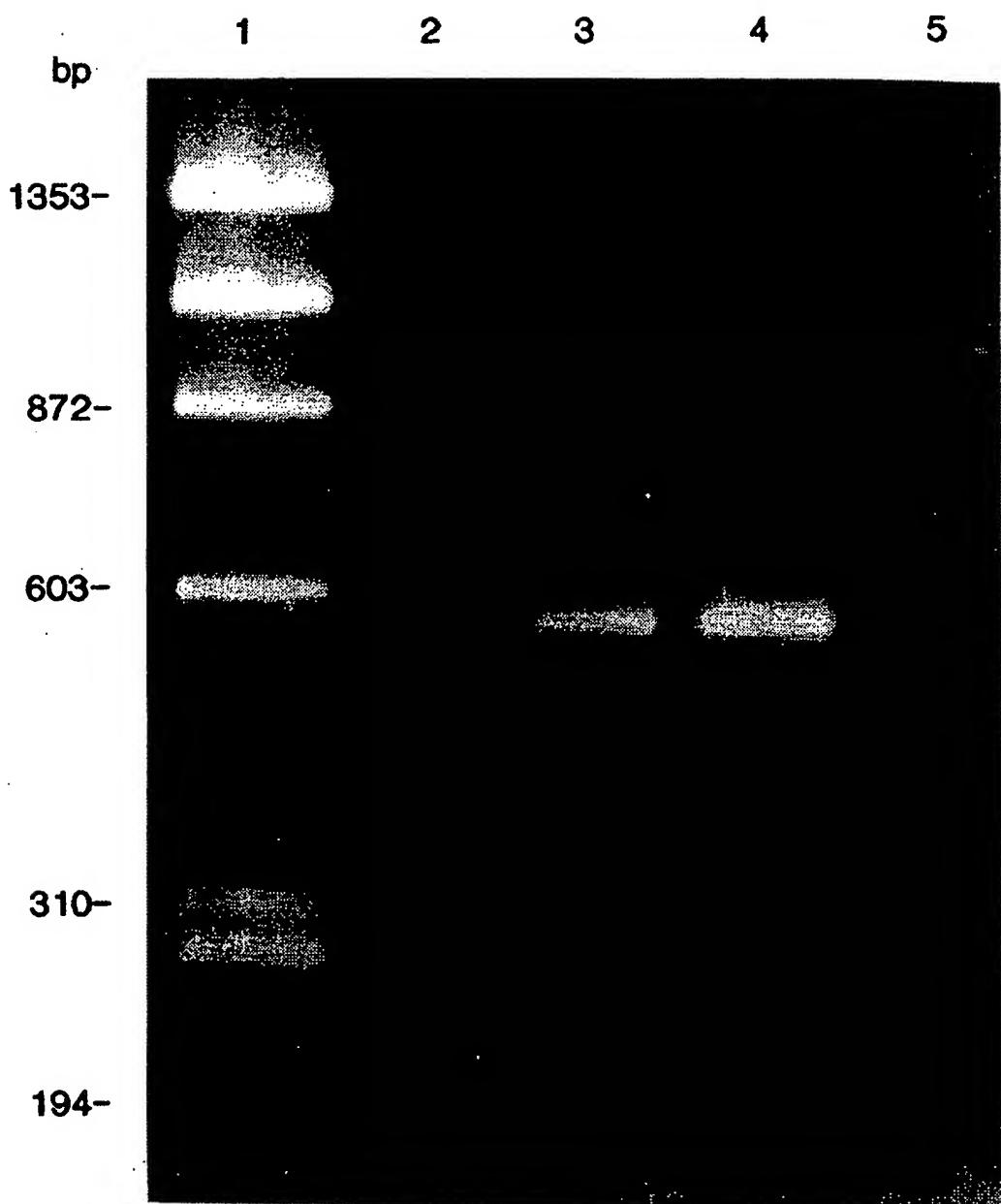


FIG. 8

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FIG. 9A

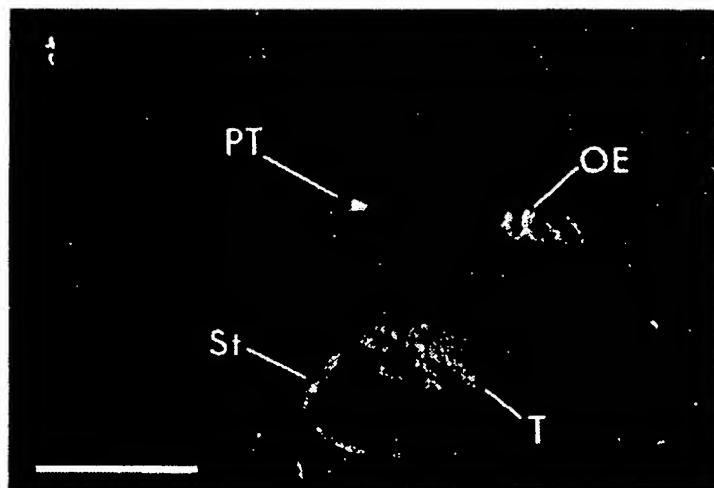


FIG. 9B

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FIG. 9C

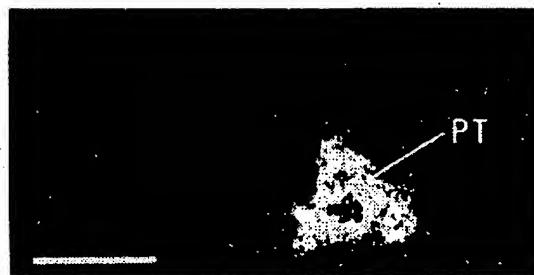
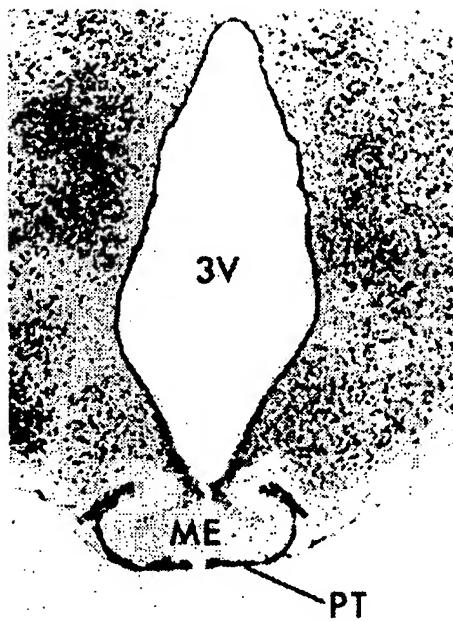
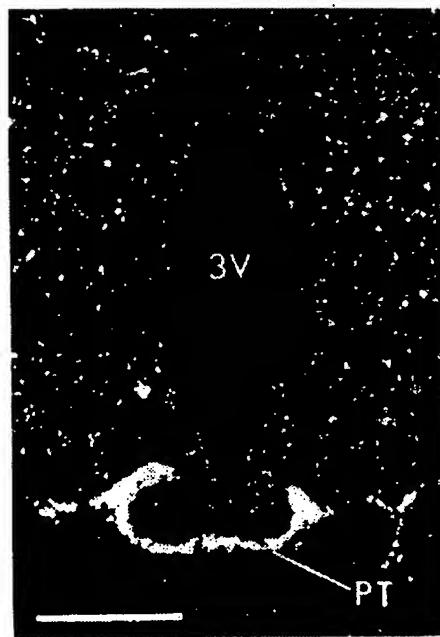


FIG. 9D

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**FIG. 9E****FIG. 9F****BEST AVAILABLE COPY**

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/09104

| | | | | | |
|-------------------------------------|-----------|------------|------------|----------|----------|
| A. CLASSIFICATION OF SUBJECT MATTER | | | | | |
| IPC 6 | C12N15/12 | C12N15/85 | C07K14/705 | C12N5/10 | C12Q1/68 |
| | C07K16/28 | A01K67/027 | C12Q1/00 | | |

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N C12Q A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|----------|--|-----------------------|
| A | <p>WO,A,91 15602 (THE SALK INSTITUTE BIOTECHNOLOGY/INDUSTRIAL ASSOCIATES, INC.) 17 October 1991 see page 5, line 5 - page 6, line 27 see page 8, line 5 - page 12, line 15 see page 13, line 34 - page 18, line 27 ----</p> <p style="text-align: center;">-/-</p> | 1-34 |

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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Date of the actual completion of the international search

14 December 1995

Date of mailing of the international search report

28.12.95

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Fax (+ 31-70) 340-3016

Authorized officer

Montero Lopez, B

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|----------|---|-----------------------|
| P, X | <p>CELL (CAMBRIDGE, MASS.) (1994), 79(4), 705-15 CODEN: CELLB5; ISSN: 0092-8674, 1994</p> <p>ELGOYHEN, ANA B. ET AL 'Acetylcholine receptor. alpha. 9 with novel pharmacological properties expressed in rat cochlear hair cells' see the summary see page 705, right column, paragraph 4 - page 706, left column, paragraph 1 see page 711, left column, paragraph 1 see page 713, left column, paragraph 7 -----</p> | 1-9, 12, 29 |

INTERNATIONAL SEARCH REPORTInternational Application No
PCT/US 95/09104

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---------------------|--------------------------------------|----------------------|
| WO-A-9115602 | 17-10-91 | US-A- EP-A- 5369028 0523187 | 29-11-94 20-01-93 |